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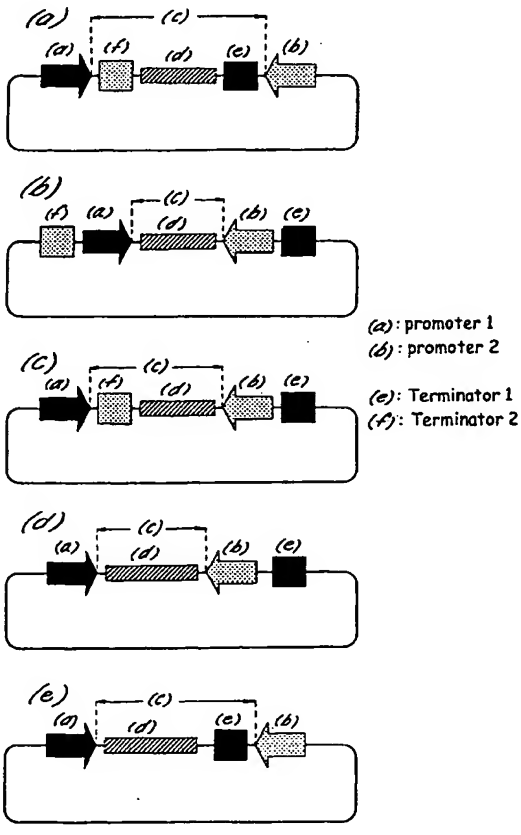

(57) Abstract: Vector constructs useful in the expression of double-stranded RNA. The constructs are particularly useful for expression of double-stranded RNA in vitro and in vivo.

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VECTOR CONSTRUCTS

Field of the invention

The invention relates to improved vector constructs for use in the expression of double-stranded RNA, particularly for use in the expression of double-stranded RNA *in vitro* and *in vivo*.

Background to the invention

Since the advent of double-stranded RNA inhibition (RNAi) as a tool for controlling gene expression, as described in WO 99/32619 and WO 00/01846, there has been recognised a need for specialised vectors designed for the production of double-stranded RNA (dsRNA).

Cloning vectors designed to produce high levels of dsRNA have been previously described by Plaetinck et al. (WO 00/01846) and Timmons et al. Nature, 395:854 (1998). These vectors generally contain a multiple cloning site (MCS) into which target DNA fragments can be cloned flanked by two opposable transcriptional promoters. Essentially, these three components (Promoter 1, MCS and Promoter 2) make up the entire system. In the appropriate expression system, the DNA cloned into the MCS may be transcribed in both directions, leading to the production of two complementary RNA strands.

A disadvantage of the known systems is that not only the cloned fragment is transcribed. Read-through of the RNA polymerase will result in transcription of the entire vector, and this also in both directions. As only transcription of the cloned DNA fragment will result in active dsRNA for RNAi purposes, transcription of the vector part results in useless,

inefficient RNA. More specifically, 80% of these transcripts can be considered as non-specific and thus non-effective.

The large amounts of non-specific RNA generated by the prior art plasmid and expression systems results in some undesirable side effects. First, in RNAi protocols based on introduction of dsRNA into *C. elegans* via a food organism such as *E. coli* which expresses the dsRNA (see WO 00/01846), large RNA strands are considered to be toxic for the food organism. As a result, high amounts of RNA accumulating in *E. coli* cause a significant part of the population to die. Second, and probably more important, is the reduction of inhibition potential. The presence of large amounts of non-specific dsRNA causes a competitive environment for the specified sequences. The potential of the template-specified dsRNA sequences to inhibit the targeted protein expression in, for instance, *C. elegans* cells is reduced by the presence of these large non-specific regions. Such an inhibition by non-specific dsRNA has also been shown in Drosophila by Tushl et al., Genes & Development 13:3191-3197 (1999). Not only the potential to inhibit gene expression is affected, but also the amount of specific dsRNA produced is limited. Third, transcription of the vector backbone part, more particularly transcription of the origin of replication and related structures, results in plasmid instability and plasmid reorganisation, leading to reduced production of dsRNA. This relatively low concentration of effective dsRNA in turn leads to inefficient RNAi.

To conclude, the previously described vectors have following shortcomings: they are toxic to the

feeding organism, a greater proportion of the transcripts produced are non-specific, the inhibitory potential of the dsRNA is reduced by the presence of non-specific regions, a high incidence of plasmid reorganizations and loss of plasmid from the feeding organism. It is therefore an object of the present invention to provide improved vectors for the production of dsRNA which avoid the disadvantages of the prior art vectors.

Vectors for use in the *in vitro* synthesis of RNA transcripts, for example the production of RNA probes, have been known and commonly used in the art for some time (see for example F. M. Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994); Jendrisak et al, Vectors for in vitro production of RNA copies of either strand of a cloned DNA sequence, US 4,766072). In standard *in vitro* transcription protocols the problem of read-through transcription of vector sequences is generally avoided by linearizing the transcription vector at restriction site positioned at the 3' end of the desired transcript. However, this solution is not appropriate for *in vivo* transcription or for the production of dsRNA where it is important that the template is transcribed in both directions.

The inventors now propose a novel solution to the problems encountered with the prior art vectors for the production of dsRNA, based on the use of transcription terminators. Generally the solution consists of the use of at least one transcription terminator operably linked to at least one promoter, wherein the terminator stops the transcription initiated by the promoter. Any DNA fragment inserted between the 3'end of the promoter and the 5' end of

the terminator will then be transcribed, without the unwanted transcription of the vector backbone. Preferentially the vector consists of two promoters and two terminators, as further described below.

Therefore, in accordance with a first aspect of the invention there is provided a DNA construct comprising two opposable promoters flanking an inter-promoter region, the construct further comprising at least one transcription terminator positioned transcriptionally downstream of one of the said promoters In particular, the invention provides for: a DNA construct comprising:

a) a first promoter and

b) a second promoter,

in which the first and second promoter are in opposite orientation to each other and define:

c) an inter-promoter region positioned downstream of the 3′ end of the first promoter and downstream of the 3′ end of the second promoter;

and which DNA construct further comprises:

d) at least one cloning site positioned in the inter-promoter region; and

e) a first transcription terminator, positioned (as seen from the 3′ end of the first promoter) downstream of the first promoter and downstream of the at least one cloning site, wherein the first transcription terminator is operably linked to the first promoter.

The inter-promoter region can also further be defined as: the DNA region between the 3′ end of the first promoter and the 3′ end of the second promoter, and which is downstream of the first promoter, and which is downstream of the second promoter, and which preferably does not contains the 5′ end of the first

promoter and of the second promoter. The opposable first promoter and second promoter drive expression directional from their 5′ ends to their 3′ ends starting transcription downstream of their 3′ ends, thus providing transcription of both strands of any nucleotide sequence(s) present in the inter-promoter region.

The two promoters present in the DNA construct of the invention may be identical or they may be different and may be of essentially any type. The precise nature of the promoters used in the construct may be dependent on the nature of the expression system in which the construct is expected to function (e.g. prokaryotic vs eukaryotic host cell). Bacteriophage promoters, for example the T7, T3 and SP6 promoters, are preferred for use in the constructs of the invention, since they provide advantages of high level transcription which is dependent only on binding of the appropriate RNA polymerase. Each of these promoters can independently by chosen. The phage promoters can also function in a wide variety of host systems, i.e. both prokaryotic and eukaryotic hosts, provided that the cognate polymerase is present in the host cell.

The arrangement of two "opposable" promoters flanking an inter-promoter region such that transcription initiation driven by one of the promoters results in transcription of the sense strand of the inter-promoter region and transcription initiation driven by the other promoter results in transcription of the antisense strand of the inter-promoter region is an arrangement well known in the art, for example, in the pGEM7 series of vectors from Promega Corp., Madison WI, USA.

The DNA constructs of the invention differ from those of the prior art because of the presence of at least one transcription terminator positioned transcriptionally downstream of one of the promoters. The transcription terminator may be uni- or bi-directional, the choice of uni- vs bi-directional terminators being influenced by the positioning of the terminator(s) within or outside the inter-promoter region, as explained below. The terminator may be of prokaryotic, eukaryotic or phage origin. Bacteriophage terminators, for example T7, T3 and SP6 terminators, are particularly preferred. The only requirement is that the terminator must be capable of causing termination of transcription initiating at the promoter relative to which it is transcriptionally downstream. In practice, these means that the promoter and terminator must form a 'functional combination', i.e. the terminator must be functional for the type of RNA polymerase initiating at the promoter. By way of example, a eukaryotic RNA pol II promoter and a eukaryotic RNA pol II terminator would generally form a functional combination. The selection of a functional combination is particularly important where bacteriophage promoters and terminators are to be used in the constructs of the invention, since the phage promoters and terminators are both polymerase-specific. To form a functional combination both the promoter and the terminator should be specific for the same polymerase, e.g. T7 promoter and T7 terminator, T3 promoter and T3 terminator etc.

In one embodiment, the DNA construct of the invention may comprise a single transcription terminator, positioned (as seen from the 3' end of the

first promoter) downstream of the first promoter and downstream of the at least one cloning site, wherein the first transcription terminator is operably linked to the first promoter, wherein the single transcription terminator is positioned in the inter-promoter region

In an alternative arrangement, the DNA construct comprises a single transcription terminator positioned outside of the inter-promoter region. In a still further embodiment, the DNA construct may comprise two transcription terminators, each one of which is positioned transcriptionally downstream of one of the two promoters. In this arrangement, one or both of the terminators may be positioned within the inter-promoter region. These various embodiments of the DNA constructs of the invention will be more fully described below, with reference to the accompanying drawings.The position of a first transcription terminator outside the inter-promoter region may also be further defined as, i.e. such that a first transcription terminator is positioned (as seen from the 3′ end of the first promoter) downstream of the first promoter, downstream of the at least one cloning site, and downstream of the 5′ end of the second promoter.

The position of a second transcription terminator outside the inter-promoter region may also be further defined as, i.e. such that a second transcription terminator positioned (as seen from the 3′ end of the second promoter) downstream of the second promoter, downstream of the at least one cloning site, and downstream of the 5′ end of the first promoter.

Moreover, when the terminator is not located in the inter-promoter region, the distance between the 5'

end of the first promoter and 3' end of the second terminator, or the distance between the 5' end of the second promoter and the 3' end of the first terminator is preferably small, i.e. such that the 3' end of the first transcription terminator is separated from the 5' end of the second promoter by no more than 2000 nucleotides, preferably no more than 1000 nucleotides, more preferably no more than 500 nucleotides, even more preferably no more than 200 nucleotides, especially preferably no more than 100 nucleotides, more especially preferable no more than 50 nucleotides, even more especially preferably no more than 20 nucleotides, particularly preferably no more than 10 nucleotides, more particularly preferably no more than 6 nucleotides.

Furthermore, when the second transcription terminator is located outside of the inter-promoter region, preferably the 3' end of the second transcription terminator is separated from the 5' end of the first promoter by no more than 2000 nucleotides, preferably no more than 1000 nucleotides, more preferably no more than 500 nucleotides, even more preferably no more than 200 nucleotides, especially preferably no more than 100 nucleotides, more especially preferably no more than 50 nucleotides, even more especially preferably no more than 20 nucleotides, particularly preferably no more than 10 nucleotides, more particularly preferably no more than 6 nucleotides

As defined above the term 'inter-promoter region' refers to all of the DNA sequence between the two promoters. As explained above, in certain embodiments of the invention the transcription terminator(s) may be sited within the inter-promoter region. The inter-

promoter region may, advantageously, comprise a sequence of nucleotides forming a template for dsRNA production. The precise length and nature of this sequence is not material to the invention. The invention further provides DNA constructs in which the inter-promoter region comprises a cloning site. The function of the cloning site is to facilitate insertion of a DNA fragment forming a template for dsRNA production between the two promoters. Thus, the invention provides a series of cloning vectors which are of general use in the construction of template vectors for dsRNA production. Also encompassed within the scope of the invention are vectors derived from the cloning vectors which have a DNA fragment inserted into the cloning site.

The cloning site may further comprise one or more of the following:

- at least one restriction site, (as known in the art), or one or more further restriction sites, e.g. to provide a multiple cloning site (as known in the art),
- a stuffer DNA, e.g., flanked by at least two restriction site, such as two *BstX*I restriction sites, or two *Xcm*I restriction sites,
- *att*R1 and *att*R2 recombination sites,
- a ccdB nucleotide sequence,
- a ccdB nucleotide further comprising at least one unique blunt-end restriction site, such as a *Srf*I restriction site, and/or
- a DNA fragment inserted in the at least one cloning site.

All of the DNA constructs provided by the invention may, advantageously, form part of a replicable cloning vector, such as, for example, a plasmid vector. In addition to the opposable

promoters, inter-promoter region and transcription terminator(s), the vector 'backbone' may further contain one or more of the general features commonly found in replicable vectors, for example an origin of replication to allow autonomous replication within a host cell and a selective marker, such as an antibiotic resistance gene. The selective marker gene (e.g. the antibiotic resistance gene) may itself contain a promoter and a transcription terminator and it is to be understood that these are completely independent of the promoter and terminator elements required by the invention and are not to be taken into consideration in determining whether a particular vector falls within the scope of the invention.

DNA constructs according to the invention may be easily be constructed from the component sequence elements using standard recombinant techniques well known in the art and described, for example, in F. M. Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994), as will be appreciated by one skilled in the art from the following detailed description of the invention and the accompanying Examples.

There follows a detailed description of DNA constructs according to the invention, with reference to the following schematic drawings in which:

Figures 1(a) to 1(e) are schematic representations of several different embodiments of the DNA construct according to the invention illustrating the relative positioning of the promoter and transcription terminator elements.

Figure 2(a) is a schematic representation of a prior

art vector included for comparison purposes.

Figures 2(b) to 2(e) are schematic representations of several further embodiments of the DNA construct according to the invention illustrating the use of different cloning sites in the inter-promoter region.

Referring to the Drawings, Figure 1(a) schematically illustrates a first DNA construct according to the invention which is a plasmid vector comprising two opposable promoters; a first promoter a)and and second promoter b)2 flanking an inter-promoter region c) , which inter-promoter region is downstream of the 3′ of the first promoter, and down stream of the 3′ end of the second promoter. The first promoter and the second promoter may be identical or different. This embodiment comprises a first transcription terminators e)and a second transcription terminator f)both of which are positioned within the inter-promoter region. In this embodiment, the first terminator and the second terminator are preferentially uni-directional terminators.

A DNA fragment may be inserted in the at least one cloning site d). Such fragment is subject to transcription directed by the first promoter a) and the second promoter b) (i.e. transcription of both strands), resulting in the generation of two RNA fragments which may combine to double-stranded RNA of the inserted DNA fragment (both *in vitro* and *in vivo*).

Any desired DNA sequence, such as a genomic DNA sequence, or a cDNA sequence or any other coding sequence, may be inserted in the at least one cloning site. Without being limited to any specific

explanation, it is assumed that when a) and e) form a functional combination, RNA polymerase which initiates transcription at a) will transcribe the inter-promoter region including the at least cloning site and the DNA fragment inserted in the at least cloning site and will be terminated when it reaches e). Similarly, RNA polymerase which initiates transcription at b) will transcribe the inter-promoter region including the at least one cloning site and the DNA fragment inserted in the at least one cloning site and will terminate when it reaches f). The terminators cause the RNA polymerase to pause, stop transcription and fall off the template. This prevents the unlimited transcription of the vector backbone, and reduces the unspecific transcription of non-essential DNA.

The inter-promoter region further comprises a sequence of nucleotides corresponding to a target for double-stranded RNA inhibition. This sequence is designated 'TF'' for target fragment. It is this sequence which, when transcribed into dsRNA, will be responsible for specific double-stranded RNA inhibition of a target gene. The target fragment may be formed from a fragment of genomic DNA or cDNA from the target gene. Its precise length and nucleotide sequence are not material to the invention.

In the arrangement shown in Figure 1(a) the two terminators are positioned on either side of the TF within the inter-promoter region. Each of the terminators is positioned transcriptionally downstream of one of the promoters, the fist terminator e) is transcriptionally downstream of first promoter a) and the second terminator f) is transcriptionally downstream of the second promoter b). Assuming that a) and e) form a functional combination, as described

above, RNA polymerase which initiates transcription at a) will transcribe the inter-promoter region up to and including TF and will be terminated when it reaches e). Similarly, RNA polymerase which initiates transcription at b) will transcribe the inter-promoter region up to and including TF on the opposite strand and will terminate when it reaches f). The terminators cause the RNA polymerase to pause, stop transcription and fall off the template. This prevents the unlimited transcription of the vector backbone, and reduces the unspecific transcription of non-essential DNA.

The transcripts generated from this vector may, depending on the precise placement of the terminators in the vector, be almost completely specific dsRNAs corresponding to the TF region. Through the direct placement of the terminator sequences at the downstream end of the TF region on both sides of the inserted DNA fragment, the amount of material transcribed is completely reduced to the template-specified sequences. Therefore, a higher amount of specific dsRNA is obtained. Furthermore the constructs are now also more stable, due to the non-transcription of the vector backbone. The latter characteristic (stability), combined with the now relatively higher specific transcription rate, provides a system adapted to synthesise higher amounts of specific short dsRNA strands. This proportionally higher amount of transcript, resulting in high concentrations of specific dsRNA, enhances the inhibitory effect in RNAi protocols. In RNAi protocols based on expression of dsRNA in a food organism, toxicity for the feeding organisms due to high RNA expression is brought to a minimal level by

use of this vector.

A specific example of a vector of the type illustrated in Figure 1(a), considered by the inventors to be the optimal arrangement for RNAi applications, is plasmid pGN9 described in the accompanying Examples. The transcriptional terminators used in pGN9 are T7 RNA polymerase specific terminators, since the vector contains two opposable T7 promoters. However, other systems could be used such as an expression system based on the T3 or SP6 promoter, terminator and polymerase or other prokaryotic or eukaryotic promoters and terminators.

Figure 1(b) illustrates schematically a further DNA construct according to the invention which is a plasmid vector comprising two opposable promoters a) and b) flanking an inter-promoter region c). This vector also comprises two transcription terminators e) and f) but in this arrangement the two terminators are positioned outside of the inter-promoter region, in fact the terminator elements now flank the two promoters. The arrangement is such that e)is transcriptionally downstream of a) whilst f) is transcriptionally downstream of b). Here again e) terminates the transcription initiated by a), whilst f) terminates the transcription initiated by promoter b). Placement of the terminators outside of d)allows the use of bi-directional terminators as well as uni-directional terminators, in contrast to the arrangement in Figure 1(a) where uni-directional terminators are preferred because of the placement of the terminators between the promoters. A number of bi-directional terminators which could be used in accordance with the invention are known in the art.

Generally these are observed to be polymerase non-specific.

The embodiment shown in Figure 1(b) provides essentially the same advantages as that shown in Figure 1(a) over the prior art vectors for dsRNA production. The vector shown in Figure 1(b) will lead to the production of transcripts which are slightly longer, including the promoter regions. This relatively small difference in the length of the transcript and hence the formed dsRNA will not severely affect the efficacy in an RNAi system.

The position of the terminators and promoter in the example as shown in figure 1 (b) are preferably placed at close proximity, such that the 5′ end of the promoters are separated from the 3′ end of the transcription terminators by no more than 2000 nucleotides, preferably no more than 1000 nucleotides, more preferably no more than 500 nucleotides, even more preferably no more than 200 nucleotides, especially preferably no more than 100 nucleotides, more especially preferably no more than 50 nucleotides, even more especially preferably no more than 20 nucleotides, particularly preferably no more than 10 nucleotides, more particularly preferably no more than 6 nucleotides.

Figure 1(c) illustrates schematically a further DNA construct according to the invention which is a plasmid vector comprising two opposable promoters a) and b) flanking an inter-promoter region c). In this embodiment one terminator (in this case e)) is positioned within the c) and the other (f)) is positioned outside c). Again, e) terminates transcription initiated by a) and f) terminates

transcription initiated by b). This arrangement may provide a useful solution to the problem of one of the terminators interfering with polymerase activity in the other direction (e.g. f) interferes with b) initiated transcription). This vector essentially provides the same advantages as the vector variations shown in Figure 1(a) and Figure 1(b) over the prior art. The relatively small difference in the length of the transcript due to the transcription of one of the promoters will not significantly affect the efficacy in RNAi systems. This more particularly the case when the terminator that is located outside of the inter-promoter region c) is at close proximity of the promoter, as defined above.

Figures 1(d) and 1(e) illustrate schematically two further DNA constructs according to the invention which are both plasmid vectors comprising two opposable promoters a) and b) flanking an inter-promoter region c). These embodiments comprise a single terminator only. In the arrangement shown in Figure 1(d) a single terminator e) which terminates transcription from a) is placed outside of c). The placement of the terminator outside of the IPR allows the use of both a bi-directional terminator or a uni-directional terminator in this system. In the embodiment shown in Figure 1(d) e) is placed within the c). a) should therefore preferably be a uni-directional terminator.

Further embodiments of the DNA construct according to the invention are illustrated schematically in Figures 2(b) to 2(e).

These embodiments are all plasmid cloning vectors, based upon the optimal arrangement of promoters and terminators shown in Figure 1(a), and

described above, containing cloning sites to facilitate the insertion of a DNA fragment into the at least on cloning site.

These embodiments are all plasmid cloning vectors, based upon the optimal arrangement of promoters and terminators shown in Figure 1(a), containing cloning sites to facilitate the insertion of a target DNA fragment into the inter-promoter region.

Figure 2(a), which is a schematic representation of a prior art cloning vector, is included for comparison purposes. This vector comprises two opposable promoters a) and b), which may be identical or different, flanking a multi-cloning site (MCS).

Figure 2(b) illustrates a first type of plasmid cloning vector according to the invention. The vector contains a first opposable promoters a) and a second opposable promoter b) flanking an inter-promoter region. The inter-promoter region can further be defined as: the DNA region between the 3′ end of the first promoter and the 3′ end of the second promoter, and which is downstream of the first promoter, and which is downstream of the second promoter, and which preferably does not contains the 5′ end of the first promoter and of the second promoter. The inter-promoter promoter region further comprises terminators e) and f) flanking a multi-cloning site MCS. The MCS comprises at least one individual restriction sites, an preferably more than one restriction site as known in the art, any of which may be used for insertion of a DNA fragment.

Figure 2(c) illustrates a further type of plasmid cloning vector according to the invention. This vector again contains opposable promoters a) and b)

flanking an inter-promoter region comprising terminators e) and f). In this embodiment, a) and b) flank a cloning site which is adapted for facilitated cloning of PCR fragments, comprising a stuffer DNA flanked by two identical restriction sites, in this case BstXI sites. The specific sequence of the stuffer DNA is not essential, provided that said stuffer DNA does not interfere with the desired effect and/or the desired activity of the DNA constructs of the invention. A specific example of a vector according to this aspect of the invention described herein is plasmid pGN29.

The cloning of PCR products using BstXI recognition sites and BstXI adaptors is generally known in the art. The BstX1 adaptors are commercially obtained, such as from Invitrogen (Groningen, the Netherlands). These adaptors are non-palindromic adapters designed for easier and efficient cloning of PCR products into vectors. These use of these adaptors reduces the concatemerization of adapters or insert DNA by having non-complementary (CACA) overhangs. The stuffer DNA is included merely to allow easy differentiation between BstXI cut and uncut vector on the basis of size. Its precise length and sequence are not of importance.

Figure 2(d) illustrates a further type of plasmid cloning vector according to the invention. This vector again contains opposable promoters a) and b) flanking an inter-promoter region comprising terminators e) and f). In this embodiment, a) and b) flank a cloning site which facilitates "High Throughput" cloning based on homologous recombination rather than restriction enzyme digestion and ligation. As shown schematically in Figure 2(d), the cloning

site comprises *att*R1 and *att*R2 recombination sites from bacteriophage lambda flanking a gene which is lethal to *E. coli*, in this case the *ccd*B gene.

An alternative cloning method of DNA fragments into this vector,(not shown in Figure 2 (d)), consists of a variant of this vector, wherein the *ccd*B DNA sequence further comprises at least one unique restriction site, preferably the at least unique restriction site is a blunt-end restriction site, such as a *Srf*I restriction site. Insertion of a DNA fragment in the at least unique restriction, results in inactivation of the ccdB gene, and hence in inactivation of the lethal ccdB gene.

A further variant of a vector a shown in Figure 2(d) in which the attR1 and the attE2 are not present. Such a vector comprises an at least one cloning site, said at least one cloning site consisting of a ccdB sequence, said ccdB sequence further comprising at least one unique restriction site, preferably the at least unique restriction site is a blunt-end restriction site, such as a *Srf*I restriction site. Insertion of a DNA fragment in the at least unique restriction, results in inactivation of the ccdB gene, and hence in inactivation of the lethal ccdB gene.

These cloning sites comprising the ccdB nuclotide sequence and/or the attR sites (R1 and/or R2) are derived from the Gateway™ cloning system commercially available from Life Technologies, Inc. The Gateway™ cloning system has been extensively described by Hartley *et al.* in WO 96/40724 (PCT/US96/10082). A specific example of a vector according to this aspect of the invention described herein is pGN39.

Figure 2(e) and 2(f) illustrate a still further type of plasmid cloning vector according to the invention. This vector again contains opposable promoters a) and b) flanking an inter-promoter region c) comprising terminators e) and f). In the embodiment shown in Figure 2(e), e) and f) flank a cloning site which facilitates "high throughput" cloning of PCR products by TA™ cloning. This cloning site comprises a stuffer DNA flanked by two identical restriction sites for an enzyme which generates overhanging T nucleotides. In this case the restriction sites are XcmI sites, but other sites which are cleaved to generate overhanging T nucleotides could be used with equivalent effect. The overhanging T nucleotides facilitate cloning of PCR products which have a overhanging A nucleotide. This principle is known as TA™ cloning. The cut vector with overhanging T nucleotides can be "topomerized" to generate a cloning vector of the type shown schematically in Figure 2(f), by linking the enzyme topoisomerase to the overhanging T nucleotides. The resulting vector also facilitates the cloning of PCR products by the principle known as TOPO™ cloning.

Both the TOPO™ and TA™cloning systems, although not for the vectors described in this invention, are commercially available from Invitrogen. The TOPO™ cloning system has extensively been described by Shuman in WO 96/19497 (PCT/US95/16099). The TA™ cloning system has extensively been described by Hernstadt et al. in WO 92/06189 (PCT/US91/07147).

It will be readily appreciated by the skilled reader that whilst Figures 2(b)-2(f) illustrate the inclusion of different cloning sites into a vector of the type illustrated in Figure 1(a), these cloning

sites could be included into any of the DNA constructs of the invention, including those illustrated schematically in Figures 1(b) to 1(e)

Application of the DNA constructs of the invention in RNAi technology.

As aforementioned, a major application of the DNA constructs/vectors of the invention is in the production of double stranded RNA for use in RNAi technology. In particular, the constructs are useful in *in vivo* RNAi protocols in the nematode worm *C. elegans*.

In *C. elegans*, RNAi has traditionally been performed by injection dsRNA into the worm. Fire *et al.* describes these methods extensively in International Application No. WO 99/32619. In short, both strands of RNA are produced *in vitro* using commercially available *in vitro* transcription kits. Both strands of RNA are allowed to form dsRNA, after which the dsRNA is injected into *C. elegans*. The new vector system developed by the present inventors is a drastic improvement on this traditional method. First, the RNA can be produced in one step, for instance by using two identical promoters such as in the vector pGN9. Second, and more important, due to the presence of terminators the transcripts, and hence the formed dsRNA, will be more specific as only the cloned target fragment will be transcribed. This will result in a more efficient RNAi.

A further method to perform RNAi experiments in *C. elegans* has been described by Plaetinck *et al.* in WO 00/01846. In this method *C. elegans* worms are fed with bacteria which produce dsRNA. The dsRNA passes the gut barrier of the worm and induces the same RNAi

as if the dsRNA has been injected. For these experiments, the preferred *E. coli* strain is HT115 (DE3), and the preferred *C. elegans* strain is nuc-1;gun-1. The improved vectors provided by the invention also improve the efficiency of RNAi in this method, as shown in the example below, as only effective dsRNA is produced.

Another method to perform RNAi has also been described by Plaetinck *et al.* in WO 00/01846. In short, this method is based on the production of dsRNA in the worm itself. This can be done by using worm promoters in the described vectors, or by using a transgenic worm expressing a polymerase specific for non-*C. elegans* promoters present in the vector, such that this polymerase drives transcription of the dsRNA. The promoters will preferentially be selected from the known bacteriophage RNA promoters, such as T7 or T3 or SP6 RNA promoters, which provide the advantage of a high level of transcription dependent only on the binding of the cognate polymerase.

Plasmid vector DNA can be introduced into the worm by several methods. First, the DNA can be introduced by the traditional injection method (Methods in Cell Biology, Vol 48, C. elegans Modern Biological Analysis of an organism, ed. by Epstein and Shakes). Second, the DNA can be introduced by DNA feeding. As has been shown by Plaetinck et al. in WO 00/01846, plasmid DNA can be introduced into the worm by feeding the worm with an *E. coli* strain that harbors the plasmid. Preferentially the *E. coli* strain is OP50, or MC1061 or HT115 (DE3) but any other strain would suit for this purpose. The *C. elegans* strain is preferentially a nuc-1 mutant strain or a nuc-1; gun-1 strain. The plasmid DNA from the *E. coli*

passes the gut barrier and is introduced into the nematode, resulting in the expression of dsRNA. As with the other RNAi methods described above, the use of the new vector system will enhance the RNAi by the production of only specific dsRNA.

The invention will be further understood with reference to the following experimental Examples, together with the following additional Figures in which:

Figure 3 is a representation (plasmid map) of pGN1.

Figure 4 is a representation (plasmid map) of pGN9.

Figure 5 illustrates the nucleotide sequence of a fragment of plasmid pGN1, annotated to show the positions of the opposable T7 promoters.

Figure 6 depicts the nucleotide sequence of the T7 transcription terminator.

Figure 7 illustrates the sequences of oligonucleotides oGN27, oGN28, oGN29 and oGN30 used to insert T7 transcription terminators into pGN1. The positions of the T7 pol terminator sequences and of various restriction sites are marked.

Figure 8 illustrates the nucleotide sequence of a fragment of plasmid pGN9, annotated to show the positions of the opposable T7 promoters and the T7 transcription terminators.

Figure 9 (a) is a representation (plasmid map) of pGN29; (b) is a representation (plasmid map) of pGN39; (c) is a representation (plasmid map) of the plasmid TopoRNAi.

Figure 10 shows the complete nucleotide sequence of plasmid pGN9.

Figure 11 shows the complete nucleotide sequence of plasmid pGN29.

Figure 12 shows the complete nucleotide sequence of plasmid pGN39.

Figure 13 shows the complete nucleotide sequence of plasmid TopoRNAi.

Figure 14 shows the complete sequence of plasmid pGN49A.

Figure 15 shows the complete sequence of plasmid pGN59A.

Figure 16 is a representation (plasmid map) of pGN49A.

Figure 17 is a representation (plasmid map) of pGN59A.

Example 1-Vector construction.

The starting point for construction of the vectors exemplified herein was plasmid pGN1. This plasmid, described in the applicant's co-pending International Application No. WO 00/01846, contains two opposable T7 promoters flanking a multi-cloning site.

Vector construction was carried out according to standard molecular biology techniques known in the art and described, for example, in F. M. Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994).

1) Construction of pGN9

pGN1 was first digested with restriction enzymes EcoRI and KpnI. Oligonucleotides oGN27 and oGN28 (Figure 7) were annealed to generate a double stranded fragment which was then ligated into the EcoRI/KpnI cut vector. The resulting plasmid was re-digested with XbaI and HindIII. Oligonucleotides oGN29 and oGN30 were annealed to generate a double-stranded fragment which was then annealed into the XbaI/HindIII cut vector. The resulting vector was designated pGN9 (Figures 4 and 10).

2) Construction of further cloning vectors

pGN29 (Figure 9(a); Figure 11) was generated by replacing the MCS in pGN9 with a stuffer DNA flanked by BstXI sites. BstXI adapters are commercially available from Invitrogen (Groningen, the Netherlands).

pGN39 (Figure 9 (b); Figure 12) generated by following steps; pGN29 was digested with BstXI. BstXI adapters (Invitrogen, Groningen, The Netherlands) were ligated to Cassette A provided by the GATEWAYTM system (Life Technologies, Inc.). Cassette A contains attR1, CmR, CcdA, CcdB, attR2. The Cassette A with the adapters where then ligated into the digested pGN29, resulting in pGN39A. pGN39A contains a unique *Srf*I site in the ccdB gene.

The TopoRNAi vector (figure 9 (c); figure 13) was generated in the following way; pGN29 was digested with BstXI. Using PCR with the primers oGN103 and oGN104 and template pCDM8 (Invitrogen, Groningen, The Netherlands), a stuffer was generated which includes XcmI sites. Onto the PCR product, BstXI adapters were ligated, and the resulting ligation product was ligated in the BstXI digested pGN29 vector resulting in the TopoRNAi vector.

oGN103:5'TACCAAGGCTAGCATGGTTTATCACTGATAAGTTGG 3'
oGN104:5'TACCAAGGCTAGCATGGGCCTGCCTGAAGGCTGC 3'

PGN49A was constructed to insert an additional unique non-blunt restriction site and to delete the CmR gene pGN39. Overlap PCR was used. A first PCR was performed with primers oGN126 and oGN127 and PGN39A as template. Using primers oGN128 an oGN129 and the same template a second fragment was generated. Overlap PCR using the resulting fragments and primers oGN126P and oGN129P resulted in a final PCR product. To this final PCR

product, BstXI adapters were ligated, and the ligation product was ligated into pGN29 digested with BstXI. The resulting vector was designated pGN49A.

A control vector was generated to test the efficiency of the pGN49A cloning vector, such vector should contain the T7 promoters, but not the T7 terminators. For this, the XbaI insert of pGN49A was isolated and cloned in pGN1 digested with the same restriction enzyme. The resulting vector was designated pGN59A.

oGN126 pGATCTGGATCCGGCTTACTAAAAGCCAGATAACAGTATGC
oGN127 GGAGACTTTATCGCTTAAGAGACGTGCACTGGCCAGGGGGATCACC
oGN128:
CCAGTGCACGTCTCTTAAGCGATAAAGTCTCCCGTGAACTTTACCCGGTGG
oGN129 pGCTGTGTATAAGGGAGCCTGACATTTATATTCCCCAG

Example 2-To illustrate the usefulness of the improved vectors in RNA.

This experiment was designed to illustrate the improved efficiency of the improved vectors of this invention in double-stranded RNA inhibition, as compared to the vectors known from the prior art. A significant increase on the efficacy of the system could be expected, as more effective dsRNA was produced and hence RNAi performed better. The experimental system for this proof of concept experiment was to measure *C. elegans* rescue at 25°C in nuc-1 / pha-1(e2123)ts *C. elegans* mutants by RNAi of sup35 using dsRNA feeding of pGN-2 (-terminator) and pGN-12 (+ terminator), with PGN-1 (empty vector) as a control and dilutor. The pha-1 ts / sup-35 mutation has extensively been described by Schnabel in WO

99/49066.

The *nuc-1* mutation in C. elegans provides for a *C. elegans* strain exhibiting better uptake abilities, such as the uptake of the dsRNA complexes than wild type *C. elegans*. This mutant is deleted in the major DNAse enzymes, and inventors have proven in earlier co-pending applications that this *C. elegans* strain results in enhanced RNAi by feeding the nematode with dsRNAs.

The pha-1(e2123)ts mutation provides a mutant *C. elegans* strain with a phenotype of survival at 15°C and lethality at 25°C. This lethality is suppressible by the inhibition of sup-35 expression. RNAi of sup-35 should thus facilitate the rescue of pha-1(e2123)ts at 25°C. The vectors of the present invention, when expressing dsRNA from sup-35, should increase the efficacy of sup-35 RNAi in rescuing pha-1(e2123)ts mutants at 25°C, compared to vectors that do not contain the terminators.

Vector pGN1 was used as empty vector. Vector pGN2 (-terminator) is a vector harboring sup-35 DNA and expressing sup-35 dsRNA when introduced in the appropriate host, the vector has no transcriptional terminators inserted. Vector pGN12 (+ terminator) is a vector as described above, containing the transcriptional terminators, and hence resulting in improved dsRNA production when introduce into an appropriate host. Thus, this vector has two unidirectional transcriptional terminators, both placed inside of the inter-promoter region, and flanking the sup-35 fragment. Use of the latter

vector was expected to increase the efficacy of the system, here meaning a better rescue (survival) of pha-1(e2123)ts mutants at 25°C.

Experimental conditions

12-well micro-titer plates were filled with approximately 2 ml of NGM agar per well.
(1 liter of NGM agar: 15g Agar, 1g peptone, 3g NaCl, 1ml cholesterol solution (5 mg/ml in EtOH), with sterile addition after autoclaving of 9.5 ml 0.1M $CaCl_2$, 9.5 ml 0.1 ml $MgSO_4$, 25 ml 1M KH_2PO_4/K_2HPO_4 buffer pH 6, Ampicillin (100 µg/l), 5ml 0,1M IPTG and 5 ml nystatin solution (dissolved 10 mg/ml in 1:1 EtOH:CH_3COONH_4 7.5 M)

The dried plates were spotted with approximately 50 µl of an overnight culture of bacteria HT115 (DE3) (Fire A, Carnegie Institution, Baltimore, MD) transformed with the plasmids. Individual nematodes at the L4 growth stage were then placed in single wells at day 1. In each well 1 nematode (P1). At day two, the P1 nematodes were placed to a new well and left to incubate for a day. The same procedure was repeated at day 3. All plates were further incubated at 25°C to allow offspring to be formed. Sup35 RNAi induced survival (rescue) was measured by counting the offspring.

Results

RNAi experiment in C. elegans nuc-1/pha-1(e2123)ts mutants by feeding with *E. coli* expressing sup-35 dsRNA.

Set up:

pGN1 as control

pGN2 (sup 35 - Term.)

pGN12 (sup 35 + Term.)

pGN2 + pGN1 dilutions 1/2, 1/4, 1/8, 1/16, 1/32

pGN12 + pGN1 dilutions 1/2, 1/4, 1/8, 1/16, 1/32

Conditions:

Incubation temperature 25°C

Readout:

Count offspring (adult hermaphrodites)

pGN1 (control)

Day 1	0	0	0	0
Day 2	0	0	0	0
Day 3	0	0	0	0

pGN2 (undiluted)

Day 1	12	4	48	32
Day 2	24	23	80	85
Day 3	5	0	9	16

pGN12 (undiluted)

Day 1	16	29	37	14
Day 2	27	22	57	2
Day 3	1	2	4	1

pGN 2+1, 1/2 dilution

Day 1	0	7	0	2
Day 2	9	10	0	3
Day 3	0	2	0	0

pGN 12+1, 1/2 dilution

Day 1	22	28	103	61
Day 2	36	45	53	40
Day 3	3	3	25	1

pGN 2+1, 1/4 dilution

Day 1	28	23	0	0
Day 2	6	3	0	0
Day 3	0	0	0	0

pGN 12+1, 1/4 dilution

Day 1	*	6	36	5
Day 2		24	55	3
Day 3				

pGN 2+1, 1/8 dilution

Day 1	0	0	4	0
Day 2	0	0	11	0
Day 3	0	0	0	0

pGN 12+1, 1/8 dilution

Day 1	31	12	16	38
Day 2	4	5	37	4
Day 3	0	0	2	1

pGN 2+1, 1/16 dilution

Day 1	0	0	0	0
Day 2	0	0	0	1 little
Day 3	0	0	0	0

pGN 12+1, 1/16 dilution

Day 1	1	0	0	0
Day 2	2	0	0	1
Day 3	0	1	1	1

pGN 2+1, 1/32 dilution

Day 1	0	0	0	0
Day 2	0	0	0	0
Day 3	0	0	0	0

pGN 12+1, 1/32 dilution

Day 1	0	0	1	0
Day 2	0	L2	3	0
Day 3	2	0	L3-L4	0

* mother died

Conclusions

As expected, worms fed by bacteria harboring pGN1, did not result in the viable offspring, due to the lethal effect of the pha-1 mutation at this temperature. Feeding the nematodes with *E. coli* harboring pGN2 or pGN12 both result in viable offspring. This is due to the feeding of the worm with dsRNA from sup-35. The remarkable difference between the two feeding experiments can be seen in the dilution series. When diluting the bacteria harboring pGN2 with bacteria harboring pGN1, the number of offspring diminishes drastically, even at a low dilution of one to two. This dilution series indicates that high levels of dsRNA are needed to have a proper RNAi induction. In the feeding experiment with bacteria harboring pGN12, significant offspring is still observed at a dilution of one to eight. This indicates that in the bacteria harboring pGN12, much more effective dsRNA is formed. This experiment clearly shows that the addition of terminator sequences in vectors to express dsRNA as described above provide a significant advantage in the generation of RNAi.

Example 3: Comparison of RNAi efficiency of vectors with and without T7 terminators(pGN49 vs pGN59)

Three different genes have been cloned in the vectors pGN49A and pGN59A. The cloning was performed by amplifying the gene fragments with PfuI DNA polymerase producing blunt ends, facilitating cloning in these vectors. These PCR fragments were cloned into the vectors digested with SrfI. Correct fragment insertion of the clones was checked by PCR. The fragments are chosen such that ds expression and RNAi results in a

lethal phenotype of the offspring. This procedure allows to compare fast and easy the efficiency of the two vectors pGN49 and pGN59 in RNAi.

plasmid	Gene (acedb)	Vector backbone5
pGW5	B0511.8	pGN49A
pGW9	C01G8.7	pGN49A
pGW11	C47B2.3	pGN49A
pGW17	B0511.8	pGN59A
pGW21	C01G8.7	pGN59A
pGW23	C47B2.3	pGN59A

All the plasmids (pGW-series)are transformed in *E.coli* AB301-105 (DE3) bacteria by standard methodology. The bacteria are then grown in LB/amp at 37°C for 14-18h. These cultures were centrifuged and the bacterial pellet dissolved in S-complete buffer containing 1mM IPTG and 100 µg/µl ampiciline.

In 96 well plates containing 100 µl S-complete (containing 1 mM IPTG and 100 µg/µl ampiciline final concentration) and 10 µl of bacteria solution, 3 nematodes were added at each well, the nematodes were at the L1 growth stage.
The plates were incubated at 25°C for 5-6 days. Each

day the plates are inspected for development of the larvae and the production of F1 offspring.

Results

The RNAi was performed in eight-fold for each constructed plasmid. The results show that when T7 terminators are inserted into the vector backbone, the expected phenotype gives a 100% occurrence. When T7 terminators are not used the reproducibility can decrease up to 50%. As in the previous experiment, the results show that the addition of terminators significantly enhances RNAi performance.

DNA fragment	B0511.8	B0511.8	C01G8.7	C01G8.7	C47B2.3	C47B2.3
Vector	pGN49A	pGN59A	PGN49A	pGN59A	pGN49A	pGN59A
Resulting plasmid	PGW5	PGW17	PGW9	PGW21	PGW11	PGW23
Percentage lethal	100	75	100	87.5	100	50
Percentage offspring	0	25	0	12.5	0	50

PCR fragment generated by the primers oGN103 and oGN104 on template pCDM8

TACCAAGGCT AGCATGGTTT ATCACTGATA AGTTGG
ATAAGTTGGT GGACATATTA TGTTTATCAG TGATAAAGTG TCAAGCATGA
CAAAGTTGCA GCCGAATACA GTGATCCGTG CCGGCCCTGG ACTGTTGAAC
GAGGTCGGCG TAGACGGTCT GACGACACGC AAACTGGCGG AACGGTTGGG
GGTGCAGCAG CCGGCGCTTT ACTGGCACTT CAGGAACAAG CGGGCGCTGC
TCGACGCACT GGCCGAAGCC ATGCTGGCGG AGAATCATAC GCTTCGGTGC
CGAGAGCCGA CGACGACTGG CGCTCATTTC TGATCGGGAA TCCCGCAGCT
TCAGGCAGGC CCATGCTAGC CTTGGTACCA GCACAATGG

Overlap PCR Fragment, which was used to generate pGN49A

gatctggatccggcttactaaaagccagataacagtatgcgtatttgcgcgctg
atttttgcggtataagaatatatactgatatgtatacccgaagtatgtcaaaaa
gaggtgtgctatgaagcagcgtattacagtgacagttgacagcgacagctatca
gttgctcaaggcatatatgatgtcaatatctccggtctggtaagcacaaccatg
cagaatgaagcccgtcgtctgcgtgccgaacgctggaaagcggaaaatcaggaa
gggatggctgaggtcgcccggtttattgaaatgaacggctcttttgctgacgag
aacagggactggtgaaatgcagtttaaggtttacacctataaaagagagagccg
ttatcgtctgtttgtggatgtacagagtgatattattgacacgcccgggcga
cggatggtgatccccctggccagtgcacgtctcttaagcgataaagtctccc
gtgaactttacccggtggtgcatatcggggatgaaagctggcgcatgatgac
caccgatatggccagtgtgccggtctccgttatcggggaagaagtggctgat
ctcagccaccgcgaaaatgacatcaaaaacgccattaacctgatgttctggg
gaatataaatgtcaggctcccttatacacagc

Claims:

1. A DNA construct comprising:

a) a first promoter and

b) a second promoter,

in which the first and second promoter are in opposite orientation to each other and define:

c) an inter-promoter region positioned downstream of the 3′ end of the first promoter and downstream of the 3′ end of the second promoter;

and which DNA construct further comprises:

d) at least one cloning site positioned in the inter-promoter region; and

e) a first transcription terminator, positioned (as seen from the 3′ end of the first promoter) downstream of the first promoter and downstream of the at least one cloning site, wherein the first transcription terminator is operably linked to the first promoter.

2. A DNA construct according to claim 1, further comprising:

f) a second transcription terminator positioned (as seen from the 3′ end of the second promoter) downstream of the second promoter and downstream of the at least one cloning site.

wherein the second transcription terminator is operably linked to the second promoter.

3. A DNA construct according to claim 1 or 2, in which the first transcription terminator is positioned in the inter-promoter region.

4. A DNA construct according to claim 1 or 2, in which the first transcription terminator is positioned (as seen from the 3′ end of the first promoter) downstream of the first promoter, downstream of the at least one cloning site, and downstream of the 5′ end of the second promoter.

5. A DNA construct according to any one of claims 2, 3 or 4, in which the second transcription terminator is positioned in the inter-promoter region.

6. A DNA construct according to any of claims 2, 3 or 4 in which the second transcription terminator is positioned (as seen from the 3′ end of the second promoter) downstream of the second promoter, downstream of the at least one cloning site, and downstream of the 5′ end of the first promoter.

7. A DNA construct according to any one of claims 4, 5 or 6, in which the 3′ end of the first transcription terminator is separated from the 5′ end of the second promoter by no more than 2000 nucleotides, preferably no more than 1000 nucleotides, more preferably no more than 500 nucleotides, even more preferably no more than 200 nucleotides, especially preferably no more than 100 nucleotides, more especially preferably no more than 50 nucleotides, even more especially preferably no more than 20 nucleotides, particularly preferably no more than 10

nucleotides, more particularly preferably no more than 6 nucleotides.

8. A DNA construct according to any one of claims 6 or 7, in which the 3′ end of the second transcription terminator is separated from the 5′ end of the first promoter by no more than 2000 nucleotides, preferably no more than 1000 nucleotides, more preferably no more than 500 nucleotides, even more preferably no more than 200 nucleotides, especially preferably no more than 100 nucleotides, more especially preferably no more than 50 nucleotides, even more especially preferably no more than 20 nucleotides, particularly preferably no more than 10 nucleotides, more particularly preferably no more than 6 nucleotides.

9. A construct according to any one of the preceding claims wherein the first and the second promoter are identical.

10. A DNA construct according to any one of the claims 1 to 7 wherein the first and the second promoter are non-identical.

11. A DNA construct according to claims 8 or 9 wherein the first promoter and the second promoter are independently chosen from T7, T3 or SP6 promoters.

12. A construct according to any one of the preceding claims wherein the cloning site comprises at

least one restriction site.

13. A DNA according to claim 11 wherein the cloning site comprises at least two restriction sites flanking a sequence of stuffer DNA.

14. A DNA construct according to claim 12 wherein the at least two restriction sites are identical.

15. A DNA construct according to any one of the claim 12 to 13 wherein the at least one restriction site or the at least two restriction sites restriction sites are *BstXI* sites.

16. A DNA construct according to any one of the claims 12 to 13 wherein the restriction sites are *XcmI* sites.

17. A DNA construct according to any one of the preceding claims wherein the cloning site further comprises *att*R1 and *att*R2 recombination sequences.

18. A DNA construct according to any one of the preceding claims wherein the cloning site further comprises a *ccdB* nucleotide sequence.

19. A DNA construct according to claim 17 wherein the *ccdB* nucleotide sequence further comprises at least one unique restriction site.

20. A DNA construct according to claim 18 wherein the at least one unique restriction site are blunt-end restriction sites.

21. A DNA construct according to claim 19 wherein the blunt-end restriction sites are *SrfI* sites.

22. A DNA according to any one of the preceding claims which further comprises:

g) a DNA fragment inserted in the at least one cloning site.

23. A DNA construct according to any one of the preceding claims which is a plasmid or vector.

24. A plasmid or vector as claimed in claim 23 having the nucleotide sequence illustrated in Figure 10, Figure 11, Figure 12, Figure 13, Figure 14, or Figure 15.

25. Use of the DNA construct according to any one of the preceding claims for the production of double-stranded RNA for RNA inhibition.

26. A bacterial strain harbouring the DNA construct according to any one of the preceding claims.

27. A bacterial strain according to claim 26, wherein said bacteria strain is an *E. coli* strain.

28. Use of the bacterial strain according to claims 26 or 27 for the production of double-stranded RNA for RNA inhibition.

FIG. 1(a)

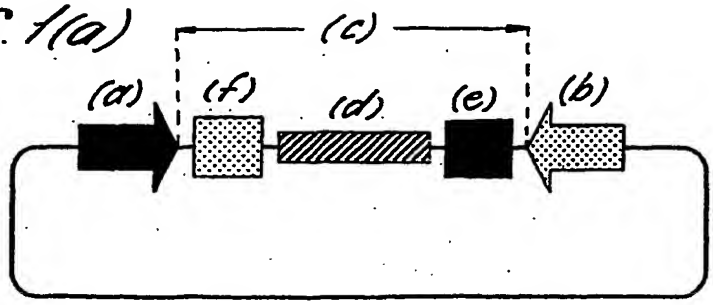
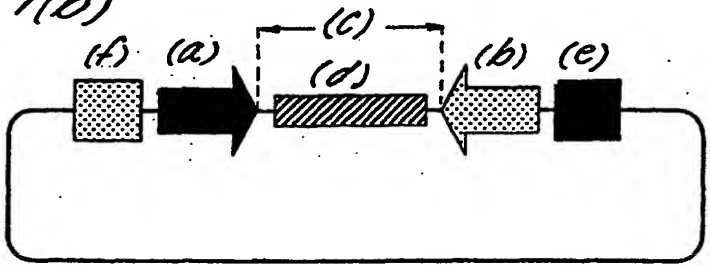


FIG. 1(b)



(a) : promoter 1
(b) : promoter 2

(e): Terminator 1
(f): Terminator 2

FIG. 1(c)

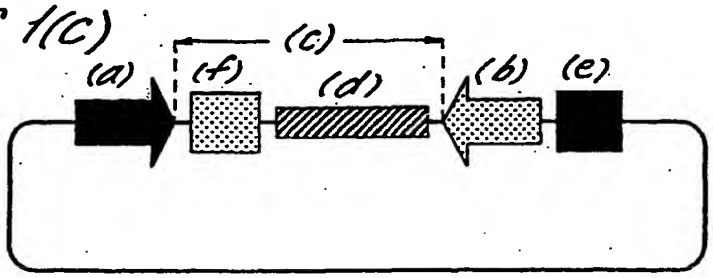


FIG. 1(d)

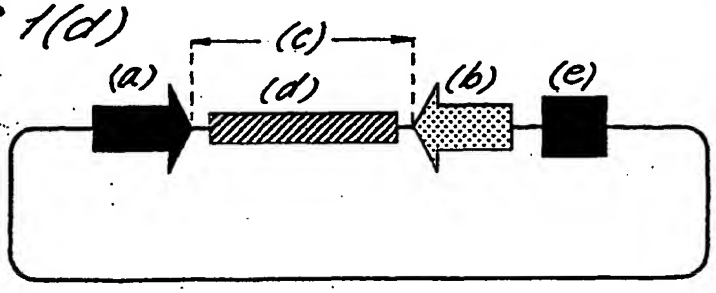


FIG. 1(e)

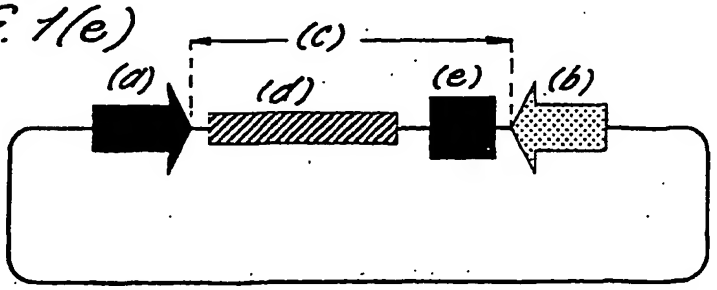


FIG. 2(a)

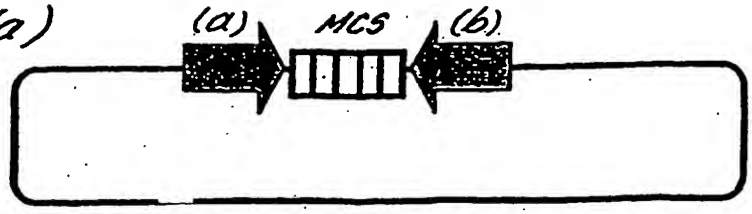


FIG. 2(b)

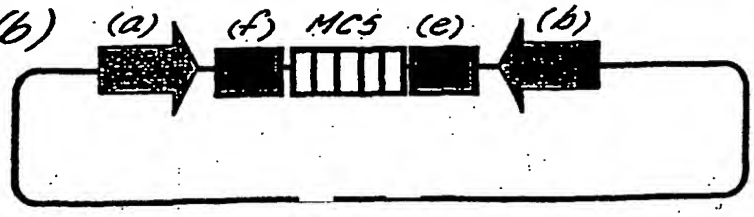


FIG. 2(c)

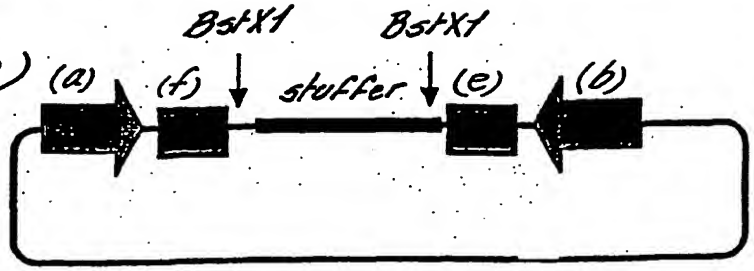


FIG. 2(d)

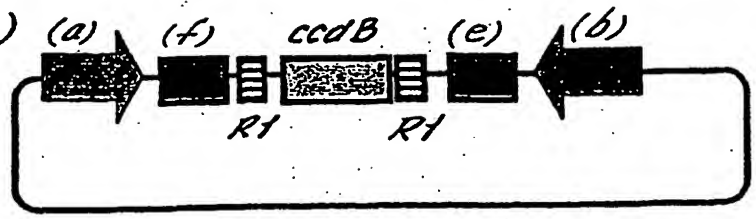


FIG. 2(e)

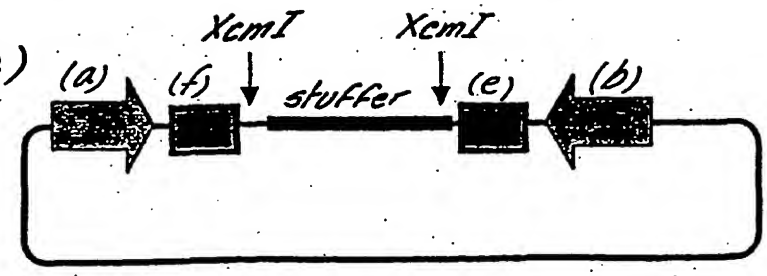
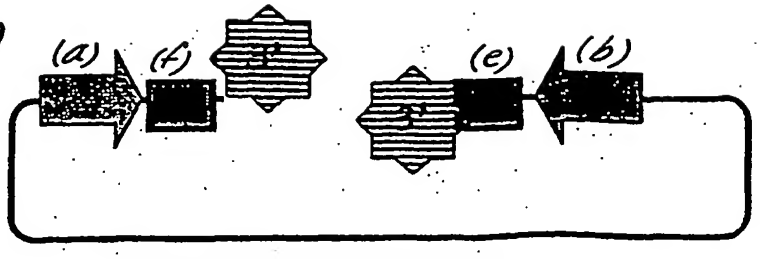


FIG. 2(f)



Construction RNAi vector with T7 terminators

FIG.3.

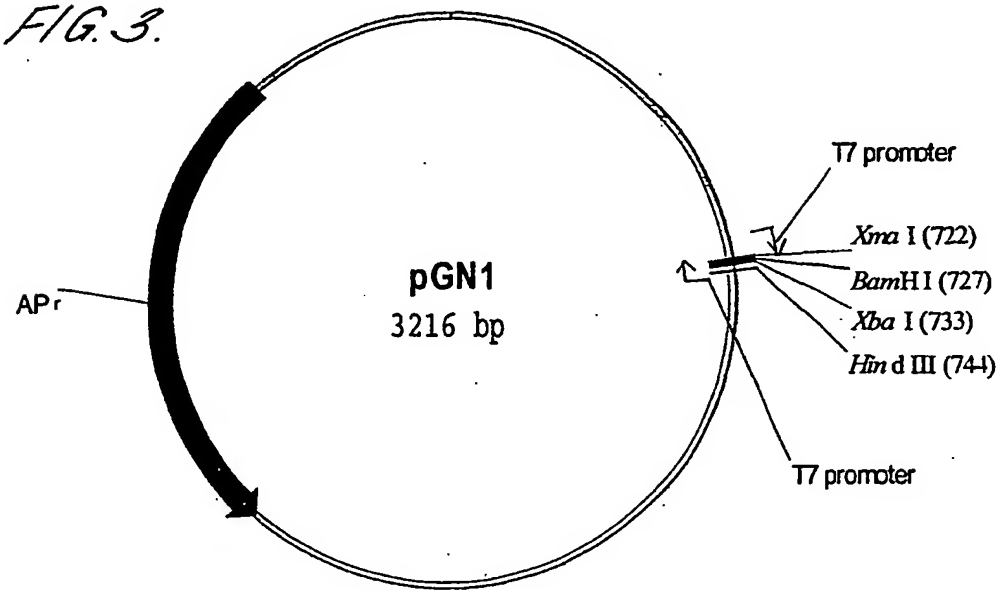


FIG.4.

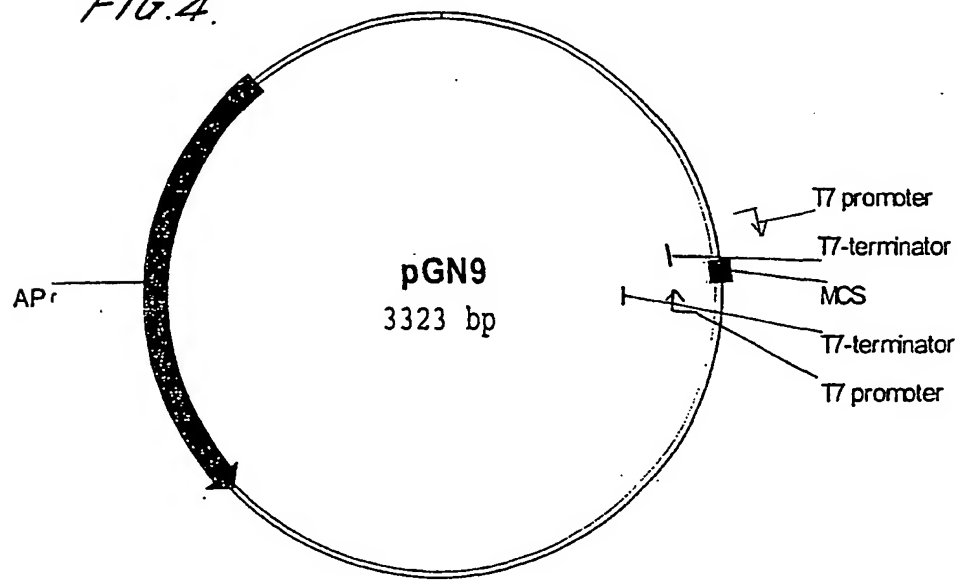
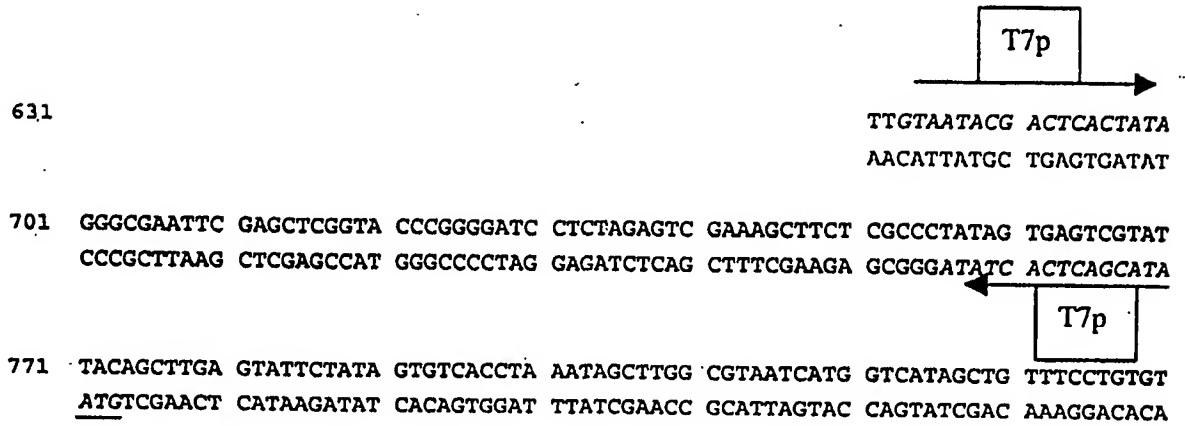


FIG. 5.


T7p
631
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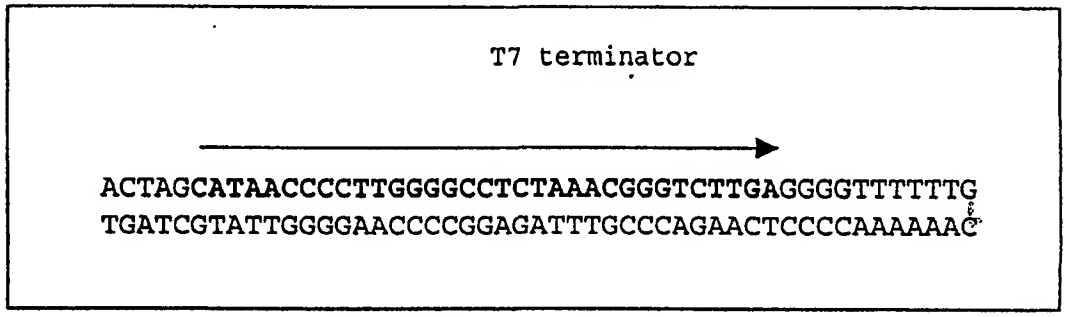
AACATTATGC TGAGTGATAT
701 GGGCGAATTC GAGCTCGGTA CCCGGGGATC CTCTAGAGTC GAAAGCTTCT CGCCCTATAG TGAGTCGTAT
CCCGCTTAAG CTCGAGCCAT GGGCCCCTAG GAGATCTCAG CTTTCGAAGA GCGGGATATC ACTCAGCATA
T7p
771 TACAGCTTGA GTATTCTATA GTGTCACCTA AATAGCTTGG CGTAATCATG GTCATAGCTG TTTCCTGTGT
ATGTCGAACT CATAAGATAT CACAGTGGAT TTATCGAACC GCATTAGTAC CAGTATCGAC AAAGGACACA

FIG. 6.


T7 terminator
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TGATCGTATTGGGGAACCCCGGAGATTTGCCCAGAACTCCCCAAAAAAC

FIG. 7.

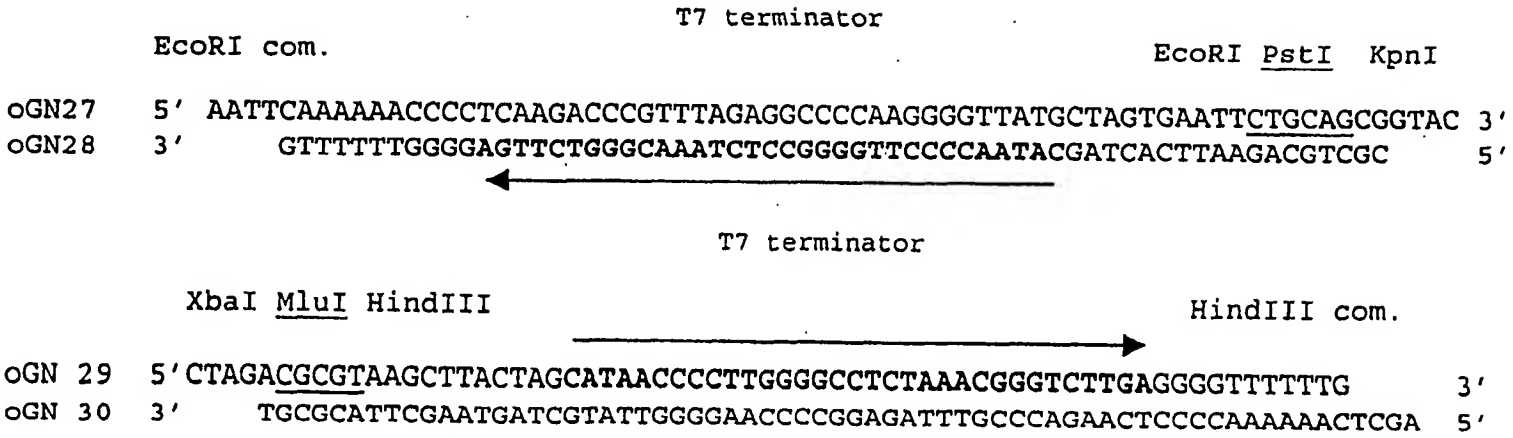
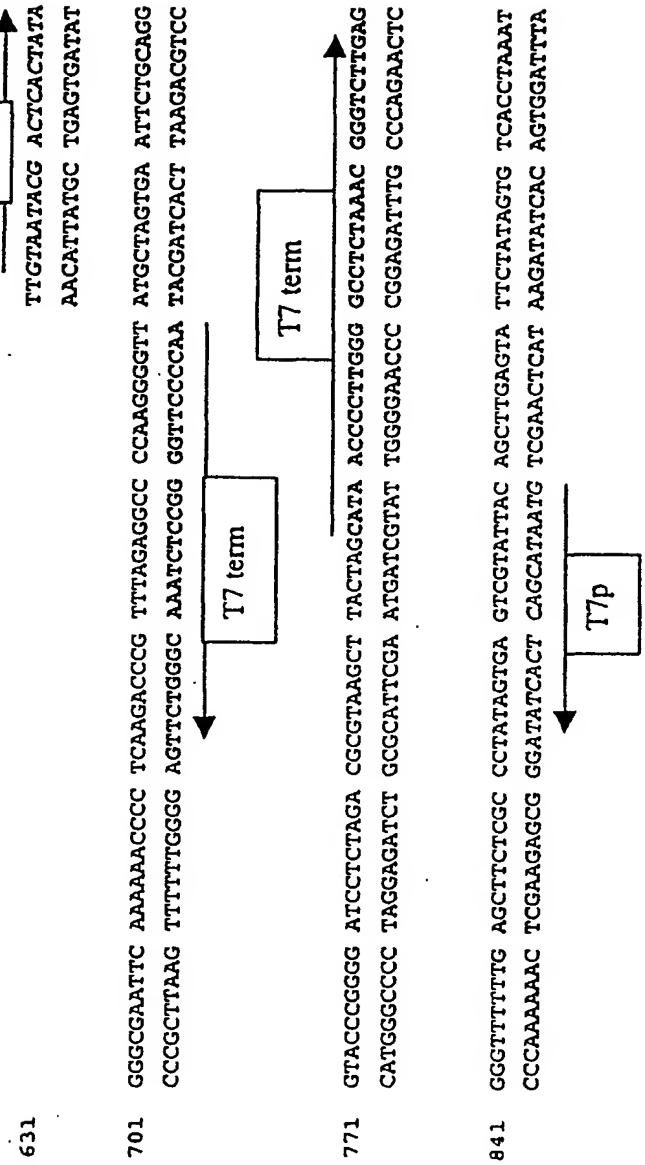

T7 terminator
EcoRI com.
EcoRI PstI KpnI
oGN27 5' AATTCAAAAAACCCCTCAAGACCCGTTTAGAGGCCCCAAGGGGTTATGCTAGTGAATTCTGCAGCGGTAC 3'
oGN28 3' GTTTTTTGGGGAGTTCTGGGCAAATCTCCGGGGTTCCCCAATACGATCACTTAAGACGTCGC 5'
T7 terminator
XbaI MluI HindIII
HindIII com.
oGN 29 5'CTAGACGCGTAAGCTTACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG 3'
oGN 30 3' TGCGCATTCGAATGATCGTATTGGGGAACCCCGGAGATTTGCCCAGAACTCCCCAAAAAACTCGA 5'

FIG. 8.


T7p
T7 term
T7 term
T7p
631
701
771
841
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AACATTATGC TGAGTGATAT
GGGCGAATTC AAAAAACCCC TCAAGACCCG TTTAGAGGCC CCAAGGGGTT ATGCTAGTGA ATTCTGCAGG
CCCGCTTAAG TTTTTTGGGG AGTTCTGGGC AAATCTCCGG GGTTCCCCAA TACGATCACT TAAGACGTCC
GTACCCGGGG ATCCTCTAGA CGCGTAAGCT TACTAGCATA ACCCCTTGGG GCCTCTAAAC GGGTCTTGAG
CATGGGCCCC TAGGAGATCT GCGCATTCGA ATGATCGTAT TGGGGAACCC CGGAGATTTG CCCAGAACTC
GGGTTTTTTG AGCTTCTCGC CCTATAGTGA GTCGTATTAC AGCTTGAGTA TTCTATAGTG TCACCTAAAT
CCCAAAAAAC TCGAAGAGCG GGATATCACT CAGCATAATG TCGAACTCAT AAGATATCAC AGTGGATTTA

FIG. 9.

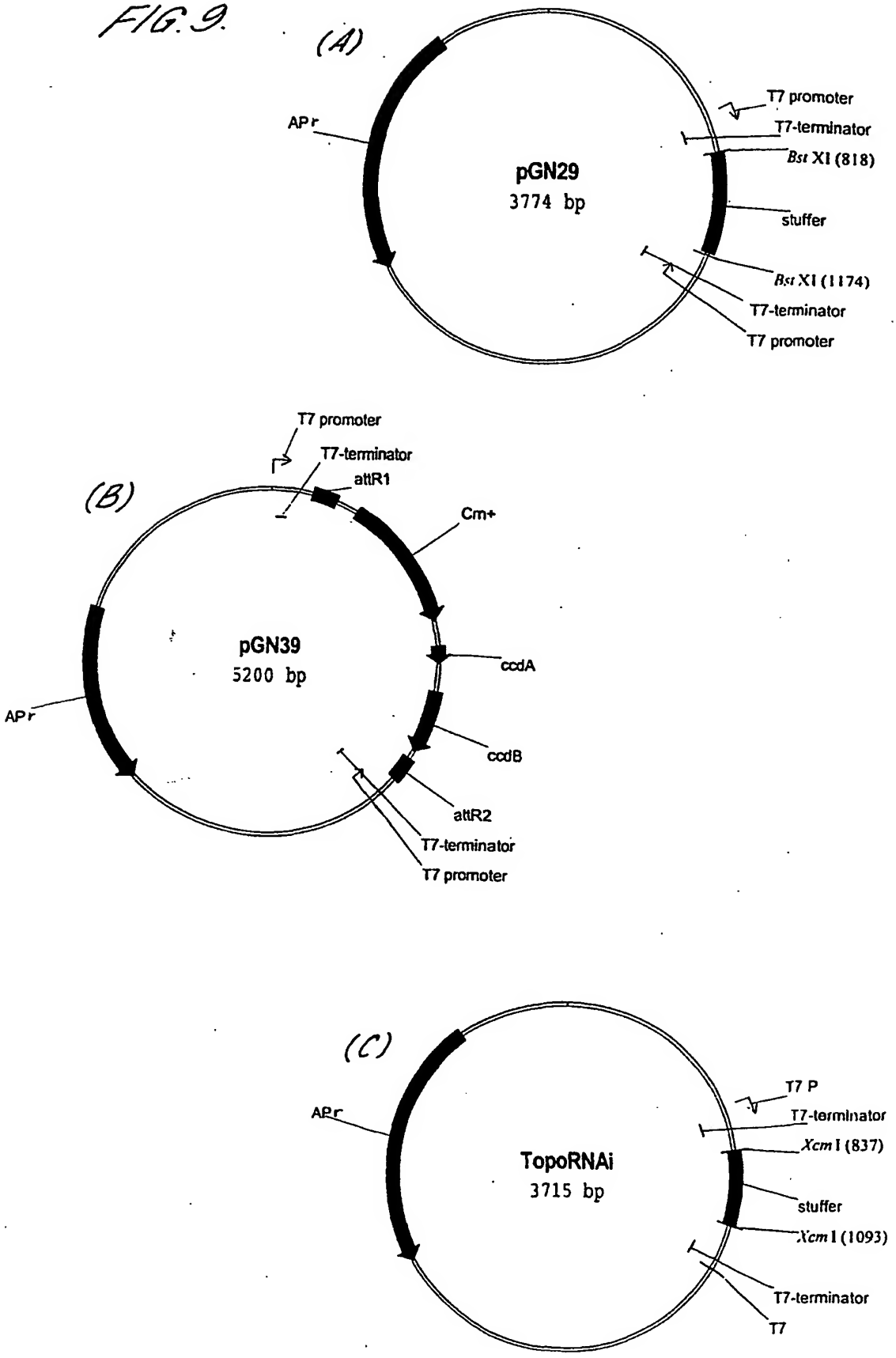
(A)
APr
pGN29
3774 bp
T7 promoter
T7-terminator
Bst XI (818)
stuffer
Bst XI (1174)
T7-terminator
T7 promoter
(B)
T7 promoter
T7-terminator
attR1
Cm+
pGN39
5200 bp
APr
ccdA
ccdB
attR2
T7-terminator
T7 promoter
(C)
APr
TopoRNAi
3715 bp
T7 P
T7-terminator
Xcm I (837)
stuffer
Xcm I (1093)
T7-terminator
T7

FIG. 10.

pGN9

```
   1 gagtgcacca tatgcggtgt gaaataccgc acagatgcgt aaggagaaaa taccgcatca
  61 ggcgaaattg taaacgttaa tattttgtta aaattcgcgt taaatatttg ttaaatcagc
 121 tcatttttta accaataggc cgaaatcggc aaaatccctt ataaatcaaa agaatagacc
 181 gagatagggt tgagtgttgt tccagtttgg aacaagagtc cactattaaa gaacgtggac
 241 tccaacgtca aagggcgaaa aaccgtctat cagggcgatg gcccactacg tgaaccatca
 301 cccaaatcaa gttttttgcg gtcgaggtgc cgtaaagctc taaatcggaa ccctaaaggg
 361 agcccccgat ttagagcttg acggggaaag ccggcgaacg tggcgagaaa ggaagggaag
 421 aaagcgaaag gagcgggcgc tagggcgctg gcaagtgtag cggtcacgct gcgcgtaacc
 481 accacacccg ccgcgcttaa tgcgccgcta cagggcgcgt ccattcgcca ttcaggctgc
 541 gcaactgttg ggaagggcga tcggtgcggg cctcttcgct attacgccag ctggcgaaag
 601 ggggatgtgc tgcaaggcga ttaagttggg taacgccagg gttttcccag tcacgacgtt
 661 gtaaaacgac ggccagtgaa ttgtaatacg actcactata gggcgaattc aaaaaacccc
 721 tcaagacccg tttagaggcc ccaaggggtt atgctagtga attctgcagg gtacccgggg
 781 atcctctaga cgcgtaagct tactagcata acccccttggg gcctctaaac gggtcttgag
 841 gggttttttg agcttctcgc cctatagtga gtcgtattac agcttgagta ttctatagtg
 901 tcacctaaat agcttggcgt aatcatggtc atagctgttt cctgtgtgaa attgttatcc
 961 gctcacaatt ccacacaaca tacgagccgg aagcataaag tgtaaagcct ggggtgccta
1021 atgagtgagc taactcacat taattgcgtt gcgctcactg cccgctttcc agtcgggaaa
1081 cctgtcgtgc cagctgcatt aatgaatcgg ccaacgcgcg gggagaggcg gtttgcgtat
1141 tgggcgctct tccgcttcct cgctcactga ctcgctgcgc tcggtcgttc ggctgcggcg
1201 agcggtatca gctcactcaa aggcggtaat acggttatcc acagaatcag gggataacgc
1261 aggaaagaac atgtgagcaa aaggccagca aaaggccagg aaccgtaaaa aggccgcgtt
1321 gctggcgttt ttcgataggc tccgcccccc tgacgagcat cacaaaaatc gacgctcaag
1381 tcagaggtgg cgaaacccga caggactata aagataccag gcgtttcccc ctggaagctc
1441 cctcgtgcgc tctcctgttc cgaccctgcc gcttaccgga tacctgtccg cctttctccc
1501 ttcgggaagc gtggcgcttt ctcatagctc acgctgtagg tatctcagtt cggtgtaggt
1561 cgttcgctcc aagctgggct gtgtgcacga accccccgtt cagcccgacc gctgcgcctt
1621 atccggtaac tatcgtcttg agtccaaccc ggtaagacac gacttatcgc cactggcagc
1681 agccactggt aacaggatta gcagagcgag gtatgtaggc ggtgctacag agttcttgaa
1741 gtggtggcct aactacggct acactagaag gacagtattt ggtatctgcg ctctgctgaa
1801 gccagttacc ttcggaaaaa gagttggtag ctcttgatcc ggcaaacaaa ccaccgctgg
1861 tagcggtggt tttttgtttt gcaagcagca gattacgcgc agaaaaaaag gatctcaaga
1921 agatcctttg atcttttcta cggggtctga cgctcagtgg aacgaaaact cacgttaagg
1981 gattttggtc atgagattat caaaaaggat cttcacctag atccttttaa attaaaaatg
2041 aagttttaaa tcaatctaaa gtatatatga gtaaacttgg tctgacagtt accaatgctt
2101 aatcagtgag gcacctatct cagcgatctg tctatttcgt tcatccatag ttgcctgact
2161 ccccgtcgtg tagataacta cgatacggga gggcttacca tctggcccca gtgctgcaat
2221 gataccgcga gacccacgct caccggctcc agatttatca gcaataaacc agccagccgg
2281 aagggccgag cgcagaagtg gtcctgcaac tttatccgcc tccatccagt ctattaattg
2341 ttgccgggaa gctagagtaa gtagttcgcc agttaatagt ttgcgcaacg ttgttggcat
2401 tgctacaggc atcgtggtgt cacgctcgtc gtttggtatg gcttcattca gctccggttc
2461 ccaacgatca aggcgagtta catgatcccc catgttgtgc aaaaaagcgg ttagctcctt
2521 cggtcctccg atcgttgtca gaagtaagtt ggccgcagtg ttatcactca tggttatggc
2581 agcactgcat aattctctta ctgtcatgcc atccgtaaga tgcttttctg tgactggtga
2641 gtactcaacc aagtcattct gagaatagcg cgcccggcga ccgagttgct cttgcccggc
2701 gtcaatacgg gataatagtg tatgacatag cagaacttta aaagtgctca tcattggaaa
2761 acgttcttcg gggcgaaaac tctcaaggat cttaccgctg ttgagatcca gttcgatgta
2821 acccactcgt gcacccaact gatcttcagc atcttttact ttcaccagcg tttctgggtg
2881 agcaaaaaca ggaaggcaaa atgccgcaaa aaagggaata agggcgacac ggaaatgttg
2941 aatactcata ctcttccttt ttcaatatta ttgaagcatt tatcagggtt attgtctcat
3001 gagcggatac atatttgaat gtatttagaa aaataaacaa atagggttc cgcgcacatt
3061 tccccgaaaa gtgccacctg acgtctaaga aaccattatt atcatgacat taacctataa
3121 aaataggcgt atcacgaggc cctttcgtct cgcgcgtttc ggtgatgacg gtgaaaacct
3181 ctgacacatg cagctcccgg agacggtcac agcttgtctg taagcggatg ccgggagcag
3241 acaagcccgt cagggcgcgt cagcgggtgt tggcgggtgt cggggctggc ttaactatgc
3301 ggcatcagag cagattgtac tga
```

FIG. 11.

PGN29

```
   1 gagtgcacca tatgcggtgt gaaataccgc acagatgcgt aaggagaaaa taccgcatca
  61 ggcgaaattg taaacgttaa tattttgtta aaattcgcgt taaatatttg ttaaatcagc
 121 tcatttttta accaataggc cgaaatcggc aaaatccctt ataaatcaaa agaatagacc
 181 gagatagggt tgagtgttgt tccagtttgg aacaagagtc cactattaaa gaacgtggac
 241 tccaacgtca aagggcgaaa aaccgtctat cagggcgatg gcccactacg tgaaccatca
 301 cccaaatcaa gtttttgcg gtcgaggtgc cgtaaagctc taaatcggaa ccctaaaggg
 361 agcccccgat ttagagcttg acggggaaag ccggcgaacg tggcgagaaa ggaagggaag
 421 aaagcgaaag gagcgggcgc tagggcgctg gcaagtgtag cggtcacgct gcgcgtaacc
 481 accacacccg ccgcgcttaa tgcgccgcta cagggcgcgt ccattcgcca ttcaggctgc
 541 gcaactgttg ggaagggcga tcggtgcggg cctcttcgct attacgccag ctggcgaaag
 601 ggggatgtgc tgcaaggcga ttaagttggg taacgccagg gttttcccag tcacgacgtt
 661 gtaaaacgac ggccagtgaa ttgtaatacg actcactata gggcgaattc aaaaaacccc
 721 tcaagacccg tttagaggcc ccaaggggtt atgctagtga attctgcagg gtacccgggg
 781 atcctctaga gatccctcga cctcgagatc cattgtgctg gcgcggattc tttatcactg
 841 ataagttggt ggacatatta tgtttatcag tgataaagtg tcaagcatga caaagttgca
 901 gccgaataca gtgatccgtg ccggccctgg actgttgaac gaggtcggcg tagacggtct
 961 gacgacacgc aaactggcgg aacggttggg ggtgcagcag ccggcgcttt actggcactt
1021 caggaacaag cgggcgctgc tcgacgcact ggccgaagcc atgctggcgg agaatcatac
1081 gcttcggtgc cgagagccga cgacgactgg cgctcatttc tgatcgggaa tcccgcagct
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1261 aacccccttgg ggcctctaaa cgggtcttga ggggtttttt gagcttctcg ccctatagtg
1321 agtcgtatta cagcttgagt attctatagt gtcacctaaa tagcttggcg taatcatggt
1381 catagctgtt tcctgtgtga attgttatc cgctcacaat tccacacaac atacgagccg
1441 gaagcataaa gtgtaaagcc tggggtgcct aatgagtgag ctaactcaca ttaattgcgt
1501 tgcgctcact gcccgctttc cagtcgggaa acctgtcgtg ccagctgcat taatgaatcg
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3541 aaaccattat tatcatgaca ttaacctata aaaataggcg tatcacgagg ccctttcgtc
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3661 cagcttgtct gtaagcggat gccgggagca gacaagcccg tcagggcgcg tcagcgggtg
3721 ttggcgggtg tcggggctgg cttaactatg cggcatcaga gcagattgta ctga
```

FIG. 12.

pGN39

TAATACGACT CACTATAGGG CGAATTCAAA AAACCCCTCA AGACCCGTTT
AGAGGCCCCA AGGGGTTATG CTAGTGAATT CTGCAGCGGT ACCCGGGGAT
CCTCTAGAGA TCCCTCGACC TCGAGATCCA TTGTGCTGGA AAGATCACAA
GTTTGTACAA AAAAGCTGAA CGAGAAACGT AAAATGATAT AAATATCAAT
ATATTAAATT AGATTTTGCA TAAAAAACAG ACTACATAAT ACTGTAAAAC
ACAACATATC CAGTCACTAT GGCGGCCGCA TTAGGCACCC CAGGCTTTAC
ACTTTATGCT TCCGGCTCGT ATAATGTGTG GATTTTGAGT TAGGATCCGG
CGAGATTTTC AGGAGCTAAG GAAGCTAAAA TGGAGAAAAA AATCACTGGA
TATACCACCG TTGATATATC CCAATGGCAT CGTAAAGAAC ATTTTGAGGC
ATTTCAGTCA GTTGCTCAAT GTACCTATAA CCAGACCGTT CAGCTGGATA
TTACGGCCTT TTTAAAGACC GTAAAGAAAA ATAAGCACAA GTTTTATCCG
GCCTTTATTC ACATTCTTGC CCGCCTGATG AATGCTCATC CGGAATTCCG
TATGGCAATG AAAGACGGTG AGCTGGTGAT ATGGGATAGT GTTCACCCTT
GTTACACCGT TTTCCATGAG CAAACTGAAA CGTTTTCATC GCTCTGGAGT
GAATACCACG ACGATTTCCG GCAGTTTCTA CACATATATT CGCAAGATGT
GGCGTGTTAC GGTGAAAACC TGGCCTATTT CCCTAAAGGG TTTATTGAGA
ATATGTTTTT CGTCTCAGCC AATCCCTGGG TGAGTTTCAC CAGTTTTGAT
TTAAACGTGG CCAATATGGA CAACTTCTTC GCCCCCGTTT TCACCATGGG
CAAATATTAT ACGCAAGGCG ACAAGGTGCT GATGCCGCTG GCGATTCAGG
TTCATCATGC CGTCTGTGAT GGCTTCCATG TCGGCAGAAT GCTTAATGAA
TTACAACAGT ACTGCGATGA GTGGCAGGGC GGGGCGTAAA GATCTGGATC
CGGCTTACTA AAAGCCAGAT AACAGTATGC GTATTTGCGC GCTGATTTTT
GCGGTATAAG AATATATACT GATATGTATA CCCGAAGTAT GTCAAAAAGA
GGTGTGCTAT GAAGCAGCGT ATTACAGTGA CAGTTGACAG CGACAGCTAT
CAGTTGCTCA AGGCATATAT GATGTCAATA TCTCCGGTCT GGTAAGCACA
ACCATGCAGA ATGAAGCCCG TCGTCTGCGT GCCGAACGCT GGAAAGCGGA
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CTTTTGCTGA CGAGAACAGG GACTGGTGAA ATGCAGTTTA AGGTTTACAC
CTATAAAAGA GAGAGCCGTT ATCGTCTGTT TGTGGATGTA CAGAGTGATA
TTATTGACAC GCCCGGGCGA CGGATGGTGA TCCCCCTGGC CAGTGCACGT
CTGCTGTCAG ATAAAGTCTC CCGTGAACTT TACCCGGTGG TGCATATCGG
GGATGAAAGC TGGCGCATGA TGACCACCGA TATGGCCAGT GTGCCGGTCT
CCGTTATCGG GGAAGAAGTG GCTGATCTCA GCCACCGCGA AAATGACATC
AAAAACGCCA TTAACCTGAT GTTCTGGGGA ATATAAATGT CAGGCTCCCT
TATACACAGC CAGTCTGCAG GTCGACCATA GTGACTGGAT ATGTTGTGTT
TTACAGTATT ATGTAGTCTG TTTTTTATGC AAAATCTAAT TTAATATATT
GATATTTATA TCATTTTACG TTTCTCGTTC AGCTTTCTTG TACAAAGTGG
TGATCTTTCC AGCACAATGG ATCTCGAGGG ATCTTCCATA CCTACCAGTT
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GCCCTATAGT GAGTCGTATT ACAGCTTGAG TATTCTATAG TGTCACCTAA
ATAGCTTGGC GTAATCATGG TCATAGCTGT TTCCTGTGTG AAATTGTTAT
CCGCTCACAA TTCCACACAA CATACGAGCC GGAAGCATAA AGTGTAAAGC

FIG. 12 (CONTINUED 1)

CTGGGGTGCC TAATGAGTGA GCTAACTCAC ATTAATTGCG TTGCGCTCAC
TGCCCGCTTT CCAGTCGGGA AACCTGTCGT GCCAGCTGCA TTAATGAATC
GGCCAACGCG CGGGGAGAGG CGGTTTGCGT ATTGGGCGCT CTTCCGCTTC
CTCGCTCACT GACTCGCTGC GCTCGGTCGT TCGGCTGCGG CGAGCGGTAT
CAGCTCACTC AAAGGCGGTA ATACGGTTAT CCACAGAATC AGGGGATAAC
GCAGGAAAGA ACATGTGAGC AAAAGGCCAG CAAAAGGCCA GGAACCGTAA
AAAGGCCGCG TTGCTGGCGT TTTTCGATAG GCTCCGCCCC CCTGACGAGC
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TAAAGATACC AGGCGTTTCC CCCTGGAAGC TCCCTCGTGC GCTCTCCTGT
TCCGACCCTG CCGCTTACCG GATACCTGTC CGCCTTTCTC CCTTCGGGAA
GCGTGGCGCT TTCTCATAGC TCACGCTGTA GGTATCTCAG TTCGGTGTAG
GTCGTTCGCT CCAAGCTGGG CTGTGTGCAC GAACCCCCCG TTCAGCCCGA
CCGCTGCGCC TTATCCGGTA ACTATCGTCT TGAGTCCAAC CCGGTAAGAC
ACGACTTATC GCCACTGGCA GCAGCCACTG GTAACAGGAT TAGCAGAGCG
AGGTATGTAG GCGGTGCTAC AGAGTTCTTG AAGTGGTGGC CTAACTACGG
CTACACTAGA AGGACAGTAT TTGGTATCTG CGCTCTGCTG AAGCCAGTTA
CCTTCGGAAA AAGAGTTGGT AGCTCTTGAT CCGGCAAACA AACCACCGCT
GGTAGCGGTG GTTTTTTTGT TTGCAAGCAG CAGATTACGC GCAGAAAAAA
AGGATCTCAA GAAGATCCTT TGATCTTTTC TACGGGGTCT GACGCTCAGT
GGAACGAAAA CTCACGTTAA GGGATTTTGG TCATGAGATT ATCAAAAAGG
ATCTTCACCT AGATCCTTTT AAATTAAAAA TGAAGTTTTA AATCAATCTA
AAGTATATAT GAGTAAACTT GGTCTGACAG TTACCAATGC TTAATCAGTG
AGGCACCTAT CTCAGCGATC TGTCTATTTC GTTCATCCAT AGTTGCCTGA
CTCCCCGTCG TGTAGATAAC TACGATACGG GAGGGCTTAC CATCTGGCCC
CAGTGCTGCA ATGATACCGC GAGACCCACG CTCACCGGCT CCAGATTTAT
CAGCAATAAA CCAGCCAGCC GGAAGGGCCG AGCGCAGAAG TGGTCCTGCA
ACTTTATCCG CCTCCATCCA GTCTATTAAT TGTTGCCGGG AAGCTAGAGT
AAGTAGTTCG CCAGTTAATA GTTTGCGCAA CGTTGTTGGC ATTGCTACAG
GCATCGTGGT GTCACGCTCG TCGTTTGGTA TGGCTTCATT CAGCTCCGGT
TCCCAACGAT CAAGGCGAGT TACATGATCC CCCATGTTGT GCAAAAAAGC
GGTTAGCTCC TTCGGTCCTC CGATCGTTGT CAGAAGTAAG TTGGCCGCAG
TGTTATCACT CATGGTTATG GCAGCACTGC ATAATTCTCT TACTGTCATG
CCATCCGTAA GATGCTTTTC TGTGACTGGT GAGTACTCAA CCAAGTCATT
CTGAGAATAC CGCGCCCGGC GACCGAGTTG CTCTTGCCCG GCGTCAATAC
GGGATAATAG TGTATGACAT AGCAGAACTT TAAAAGTGCT CATCATTGGA
AAACGTTCTT CGGGGCGAAA ACTCTCAAGG ATCTTACCGC TGTTGAGATC
CAGTTCGATG TAACCCACTC GTGCACCCAA CTGATCTTCA GCATCTTTTA
CTTTCACCAG CGTTTCTGGG TGAGCAAAAA CAGGAAGGCA AAATGCCGCA
AAAAAGGGAA TAAGGGCGAC ACGGAAATGT TGAATACTCA TACTCTTCCT
TTTTCAATAT TATTGAAGCA TTTATCAGGG TTATTGTCTC ATGAGCGGAT
ACATATTTGA ATGTATTTAG AAAAATAAAC AAATAGGGGT TCCGCGCACA
TTTCCCCGAA AAGTGCCACC TGACGTCTAA GAAACCATTA TTATCATGAC
ATTAACCTAT AAAAATAGGC GTATCACGAG GCCCTTTCGT CTCGCGCGTT
TCGGTGATGA CGGTGAAAAC CTCTGACACA TGCAGCTCCC GGAGACGGTC
ACAGCTTGTC TGTAAGCGGA TGCCGGGAGC AGACAAGCCC GTCAGGGCGC
GTCAGCGGGT GTTGGCGGGT GTCGGGGCTG GCTTAACTAT GCGGCATCAG

FIG. 12 (CONTINUED 2)

AGCAGATTGT ACTGAGAGTG CACCATATGC GGTGTGAAAT ACCGCACAGA
TGCGTAAGGA GAAAATACCG CATCAGGCGA AATTGTAAAC GTTAATATTT
TGTTAAAATT CGCGTTAAAT ATTTGTTAAA TCAGCTCATT TTTTAACCAA
TAGGCCGAAA TCGGCAAAAT CCCTTATAAA TCAAAAGAAT AGACCGAGAT
AGGGTTGAGT GTTGTTCCAG TTTGGAACAA GAGTCCACTA TTAAAGAACG
TGGACTCCAA CGTCAAAGGG CGAAAAACCG TCTATCAGGG CGATGGCCCA
CTACGTGAAC CATCACCCAA ATCAAGTTTT TTGCGGTCGA GGTGCCGTAA
AGCTCTAAAT CGGAACCCTA AAGGGAGCCC CCGATTTAGA GCTTGACGGG
GAAAGCCGGC GAACGTGGCG AGAAAGGAAG GGAAGAAAGC GAAAGGAGCG
GGCGCTAGGG CGCTGGCAAG TGTAGCGGTC ACGCTGCGCG TAACCACCAC
ACCCGCCGCG CTTAATGCGC CGCTACAGGG CGCGTCCATT CGCCATTCAG
GCTGCGCAAC TGTTGGGAAG GGCGATCGGT GCGGGCCTCT TCGCTATTAC
GCCAGCTGGC GAAAGGGGGA TGTGCTGCAA GGCGATTAAG TTGGGTAACG
CCAGGGTTTT CCCAGTCACG ACGTTGTAAA ACGACGGCCA GTGAATTG

FIG. 13.

TopoRNAi

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   1 gagtgcacca tatgcggtgt gaaataccgc acagatgcgt aaggagaaaa taccgcatca
  61 ggcgaaattg taaacgttaa tattttgtta aaattcgcgt taaatatttg ttaaatcagc
 121 tcatttttta accaataggc cgaaatcggc aaaatcccct ataaatcaaa agaatagacc
 181 gagatagggt tgagtgttgt tccagtttgg aacaagagtc cactattaaa gaacgtggac
 241 tccaacgtca aagggcgaaa aaccgtctat cagggcgatg gcccactacg tgaaccatca
 301 cccaaatcaa gttttttgcg gtcgaggtgc cgtaaagctc taaatcggaa ccctaaaggg
 361 agcccccgat ttagagcttg acggggaaag ccggcgaacg tggcgagaaa ggaagggaag
 421 aaagcgaaag gagcgggcgc tagggcgctg gcaagtgtag cggtcacgct gcgcgtaacc
 481 accacacccg ccgcgcttaa tgcgccgcta cagggcgcgt ccattcgcca ttcaggctgc
 541 gcaactgttg ggaagggcga tcggtgcggg cctcttcgct attacgccag ctggcgaaag
 601 ggggatgtgc tgcaaggcga ttaagttggg taacgccagg gttttcccag tcacgacgtt
 661 gtaaaacgac ggccagtgaa ttgtaatacg actcactata gggcgaattc aaaaaacccc
 721 tcaagacccg tttagaggcc ccaaggggtt atgctagtga attctgcagg gtacccgggg
 781 atcctctaga gatccctcga cctcgagatc cattgtggtg gaattctacc aaggctagca
 841 tgggcagccg aatacagtga tccgtgccgg ccctggactg ttgaacgagg tcggcgtaga
 901 cggtctgacg acacgcaaac tggcggaacg gttgggggtg cagcagccgg cgctttactg
 961 gcacttcagg aacaagcggg cgctgctcga cgcactggcc gaagccatgc tggcggagaa
1021 tcatacgctt cggtgccgag agccgacgac gactggcgct catttctgat cgggaatccc
1081 gcagccatgc tagccttggt agaattccac cacaatggat ctcgagggat cttccatacc
1141 taccagttct gcgcctgcag gtcgcggccg cgactctcta gacgcgtaag cttactagca
1201 taacccccttg gggcctctaa acgggtcttg aggggttttt tgagcttctc gccctatagt
1261 gagtcgtatt acagcttgag tattctatag tgtcacctaa atagcttggc gtaatcatgg
1321 tcatagctgt ttcctgtgtg aaattgttat ccgctcacaa ttccacacaa catacgagcc
1381 ggaagcataa agtgtaaagc ctggggtgcc taatgagtga gctaactcac attaattgcg
1441 ttgcgctcac tgcccgcttt ccagtcggga aacctgtcgt gccagctgca ttaatgaatc
1501 ggccaacgcg cggggagagg cggtttgcgt attgggcgct cttccgcttc ctcgctcact
1561 gactcgctgc gctcggtcgt tcggctgcgg cgagcggtat cagctcactc aaaggcggta
1621 atacggttat ccacagaatc aggggataac gcaggaaaga acatgtgagc aaaaggccag
1681 caaaaggcca ggaaccgtaa aaaggccgcg ttgctggcgt ttttcgatag gctccgcccc
1741 cctgacgagc atcacaaaaa tcgacgctca agtcagaggt ggcgaaaccc gacaggacta
1801 taaagatacc aggcgtttcc ccctggaagc tccctcgtgc gctctcctgt tccgaccctg
1861 ccgcttaccg gatacctgtc cgcctttctc ccttcgggaa gcgtggcgct ttctcatagc
1921 tcacgctgta ggtatctcag ttcggtgtag gtcgttcgct ccaagctggg ctgtgtgcac
1981 gaacccccgg ttcagcccga ccgctgcgcc ttatccggta actatcgtct tgagtccaac
2041 ccggtaagac acgacttatc gccactggca gcagccactg gtaacaggat tagcagagcg
2101 aggtatgtag gcggtgctac agagttcttg aagtggtggc ctaactacgg ctacactaga
2161 aggacagtat ttggtatctg cgctctgctg aagccagtta ccttcggaaa aagagttggt
2221 agctcttgat ccggcaaaca aaccaccgct ggtagcggtg gttttttgtt ttgcaagcag
2281 cagattacgc gcagaaaaaa aggatctcaa gaagatcctt tgatcttttc tacggggtct
2341 gacgctcagt ggaacgaaaa ctcacgttaa gggattttgg tcatgagatt atcaaaaagg
2401 atcttcacct agatcctttt aaattaaaaa tgaagtttta aatcaatcta aagtatatat
2461 gagtaaactt ggtctgacag ttaccaatgc ttaatcagtg aggcacctat ctcagcgatc
2521 tgtctatttc gttcatccat agttgcctga ctccccgtcg tgtagataac tacgatacgg
2581 gagggcttac catctggccc cagtgctgca atgataccgc gagacccacg ctcaccggct
2641 ccagatttat cagcaataaa ccagccagcc ggaagggccg agcgcagaag tggtcctgca
2701 actttatccg cctccatcca gtctattaat tgttgccggg aagctagagt aagtagttcg
2761 ccagttaata gtttgcgcaa cgttgttggc attgctacag gcatcgtggt gtcacgctcg
2821 tcgtttggta tggcttcatt cagctccggt tcccaacgat caaggcgagt tacatgatcc
2881 cccatgttgt gcaaaaaagc ggttagctcc ttcggtcctc cgatcgttgt cagaagtaag
2941 ttggccgcag tgttatcact catggttatg gcagcactgc ataattctct tactgtcatg
3001 ccatccgtaa gatgcttttc tgtgactggt gagtactcaa ccaagtcatt ctgagaatac
3061 cgcgcccggc gaccgagttg ctcttgcccg gcgtcaatac gggataatag tgtatgacat
3121 agcagaactt taaaagtgct catcattgga aaacgttctt cggggcgaaa actctcaagg
3181 atcttaccgc tgttgagatc cagttcgatg taacccactc gtgcacccaa ctgatcttca
3241 gcatctttta ctttcaccag cgtttctggg tgagcaaaaa caggaaggca aaatgccgca
3301 aaaaagggaa taagggcgac acggaaatgt tgaatactca tactcttcct ttttcaatat
3361 tattgaagca tttatcaggg ttattgtctc atgagcggat acatatttga atgtatttag
3421 aaaaataaac aaataggggt tccgcgcaca tttccccgaa aagtgccacc tgacgtctaa
3481 gaaaccatta ttatcatgac attaacctat aaaaataggc gtatcacgag gccctttcgt
3541 ctcgcgcgtt tcggtgatga cggtgaaaac ctctgacaca tgcagctccc ggagacggtc
3601 acagcttgtc tgtaagcgga tgccgggagc agacaagccc gtcagggcgc gtcagcgggt
3661 gttggcgggt gtcggggctg gcttaactat gcggcatcag agcagattgt actga
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pGN49A FIG. 14.

TGTAATACGA CTCACTATAG GGCGAATTCA AAAAACCCCT CAAGACCCGT
TTAGAGGCCC CAAGGGGTTA TGCTAGTGAA TTCTGCAGCG GTACCCGGGG
ATCCTCTAGA GATCCCTCGA CCTCGAGATC CATTGTGCTG GAAAGGATCT
GGATCCGGCT TACTAAAAGC CAGATAACAG TATGCGTATT TGCGCGCTGA
TTTTTGCGGT ATAAGAATAT ATACTGATAT GTATACCCGA AGTATGTCAA
AAAGAGGTGT GCTATGAAGC AGCGTATTAC AGTGACAGTT GACAGCGACA
GCTATCAGTT GCTCAAGGCA TATATGATGT CAATATCTCC GGTCTGGTAA
GCACAACCAT GCAGAATGAA GCCCGTCGTC TGCGTGCCGA ACGCTGGAAA
GCGGAAAATC AGGAAGGGAT GGCTGAGGTC GCCCGGTTTA TTGAAATGAA
CGGCTCTTTT GCTGACGAGA ACAGGGACTG GTGAAATGCA GTTTAAGGTT
TACACCTATA AAAGAGAGAG CCGTTATCGT CTGTTTGTGG ATGTACAGAG
TGATATTATT GACACGCCCG GGCGACGGAT GGTGATCCCC CTGGCCAGTG
CACGTCTCTT AAGCGATAAA GTCTCCCGTG AACTTTACCC GGTGGTGCAT
ATCGGGGATG AAAGCTGGCG CATGATGACC ACCGATATGG CCAGTGTGCC
GGTCTCCGTT ATCGGGGAAG AAGTGGCTGA TCTCAGCCAC CGCGAAAATG
ACATCAAAAA CGCCATTAAC CTGATGTTCT GGGGAATATA AATGTCAGGC
TCCCTTATAC ACAGCCTTTC CAGCACAATG GATCTCGAGG GATCTTCCAT
ACCTACCAGT TCTGCGCCTG CAGGTCGCGG CCGCGACTCT AGACGCGTAA
GCTTACTAGC ATAACCCCTT GGGGCCTCTA AACGGGTCTT GAGGGGTTTT
TTGAGCTTCT CGCCCTATAG TGAGTCGTAT TACAGCTTGA GTATTCTATA
GTGTCACCTA AATAGCTTGG CGTAATCATG GTCATAGCTG TTTCCTGTGT
GAAATTGTTA TCCGCTCACA ATTCCACACA ACATACGAGC CGGAAGCATA
AAGTGTAAAG CCTGGGGTGC CTAATGAGTG AGCTAACTCA CATTAATTGC
GTTGCGCTCA CTGCCCGCTT TCCAGTCGGG AAACCTGTCG TGCCAGCTGC
ATTAATGAAT CGGCCAACGC GCGGGGAGAG GCGGTTTGCG TATTGGGCGC
TCTTCCGCTT CCTCGCTCAC TGACTCGCTG CGCTCGGTCG TTCGGCTGCG
GCGAGCGGTA TCAGCTCACT CAAAGGCGGT AATACGGTTA TCCACAGAAT
CAGGGGATAA CGCAGGAAAG AACATGTGAG CAAAAGGCCA GCAAAAGGCC
AGGAACCGTA AAAAGGCCGC GTTGCTGGCG TTTTTCGATA GGCTCCGCCC
CCCTGACGAG CATCACAAAA ATCGACGCTC AAGTCAGAGG TGGCGAAACC
CGACAGGACT ATAAAGATAC CAGGCGTTTC CCCCTGGAAG CTCCCTCGTG
CGCTCTCCTG TTCCGACCCT GCCGCTTACC GGATACCTGT CCGCCTTTCT
CCCTTCGGGA AGCGTGGCGC TTTCTCATAG CTCACGCTGT AGGTATCTCA
GTTCGGTGTA GGTCGTTCGC TCCAAGCTGG GCTGTGTGCA CGAACCCCCC
GTTCAGCCCG ACCGCTGCGC CTTATCCGGT AACTATCGTC TTGAGTCCAA
CCCGGTAAGA CACGACTTAT CGCCACTGGC AGCAGCCACT GGTAACAGGA
TTAGCAGAGC GAGGTATGTA GGCGGTGCTA CAGAGTTCTT GAAGTGGTGG
CCTAACTACG GCTACACTAG AAGGACAGTA TTTGGTATCT GCGCTCTGCT
GAAGCCAGTT ACCTTCGGAA AAAGAGTTGG TAGCTCTTGA TCCGGCAAAC
AAACCACCGC TGGTAGCGGT GGTTTTTTTG TTTGCAAGCA GCAGATTACG
CGCAGAAAAA AAGGATCTCA AGAAGATCCT TTGATCTTTT CTACGGGGTC
TGACGCTCAG TGGAACGAAA ACTCACGTTA AGGGATTTTG GTCATGAGAT
TATCAAAAAG GATCTTCACC TAGATCCTTT TAAATTAAAA ATGAAGTTTT

FIG. 14 (CONTINUED)

AAATCAATCT AAAGTATATA TGAGTAAACT TGGTCTGACA GTTACCAATG
CTTAATCAGT GAGGCACCTA TCTCAGCGAT CTGTCTATTT CGTTCATCCA
TAGTTGCCTG ACTCCCCGTC GTGTAGATAA CTACGATACG GGAGGGCTTA
CCATCTGGCC CCAGTGCTGC AATGATACCG CGAGACCCAC GCTCACCGGC
TCCAGATTTA TCAGCAATAA ACCAGCCAGC CGGAAGGGCC GAGCGCAGAA
GTGGTCCTGC AACTTTATCC GCCTCCATCC AGTCTATTAA TTGTTGCCGG
GAAGCTAGAG TAAGTAGTTC GCCAGTTAAT AGTTTGCGCA ACGTTGTTGG
CATTGCTACA GGCATCGTGG TGTCACGCTC GTCGTTTGGT ATGGCTTCAT
TCAGCTCCGG TTCCCAACGA TCAAGGCGAG TTACATGATC CCCCATGTTG
TGCAAAAAAG CGGTTAGCTC CTTCGGTCCT CCGATCGTTG TCAGAAGTAA
GTTGGCCGCA GTGTTATCAC TCATGGTTAT GGCAGCACTG CATAATTCTC
TTACTGTCAT GCCATCCGTA AGATGCTTTT CTGTGACTGG TGAGTACTCA
ACCAAGTCAT TCTGAGAATA CCGCGCCCGG CGACCGAGTT GCTCTTGCCC
GGCGTCAATA CGGGATAATA GTGTATGACA TAGCAGAACT TTAAAAGTGC
TCATCATTGG AAAACGTTCT TCGGGGCGAA AACTCTCAAG GATCTTACCG
CTGTTGAGAT CCAGTTCGAT GTAACCCACT CGTGCACCCA ACTGATCTTC
AGCATCTTTT ACTTTCACCA GCGTTTCTGG GTGAGCAAAA ACAGGAAGGC
AAAATGCCGC AAAAAAGGGA ATAAGGGCGA CACGGAAATG TTGAATACTC
ATACTCTTCC TTTTTCAATA TTATTGAAGC ATTTATCAGG GTTATTGTCT
CATGAGCGGA TACATATTTG AATGTATTTA GAAAAATAAA CAAATAGGGG
TTCCGCGCAC ATTTCCCCGA AAAGTGCCAC CTGACGTCTA AGAAACCATT
ATTATCATGA CATTAACCTA TAAAAATAGG CGTATCACGA GGCCCTTTCG
TCTCGCGCGT TTCGGTGATG ACGGTGAAAA CCTCTGACAC ATGCAGCTCC
CGGAGACGGT CACAGCTTGT CTGTAAGCGG ATGCCGGGAG CAGACAAGCC
CGTCAGGGCG CGTCAGCGGG TGTTGGCGGG TGTCGGGGCT GGCTTAACTA
TGCGGCATCA GAGCAGATTG TACTGAGAGT GCACCATATG CGGTGTGAAA
TACCGCACAG ATGCGTAAGG AGAAAATACC GCATCAGGCG AAATTGTAAA
CGTTAATATT TTGTTAAAAT TCGCGTTAAA TATTTGTTAA ATCAGCTCAT
TTTTTAACCA ATAGGCCGAA ATCGGCAAAA TCCCTTATAA ATCAAAAGAA
TAGACCGAGA TAGGGTTGAG TGTTGTTCCA GTTTGGAACA AGAGTCCACT
ATTAAAGAAC GTGGACTCCA ACGTCAAAGG GCGAAAAACC GTCTATCAGG
GCGATGGCCC ACTACGTGAA CCATCACCCA AATCAAGTTT TTTGCGGTCG
AGGTGCCGTA AAGCTCTAAA TCGGAACCCT AAAGGGAGCC CCCGATTTAG
AGCTTGACGG GGAAAGCCGG CGAACGTGGC GAGAAAGGAA GGGAAGAAAG
CGAAAGGAGC GGGCGCTAGG GCGCTGGCAA GTGTAGCGGT CACGCTGCGC
GTAACCACCA CACCCGCCGC GCTTAATGCG CCGCTACAGG GCGCGTCCAT
TCGCCATTCA GGCTGCGCAA CTGTTGGGAA GGGCGATCGG TGCGGGCCTC
TTCGCTATTA CGCCAGCTGG CGAAAGGGGG ATGTGCTGCA AGGCGATTAA
GTTGGGTAAC GCCAGGGTTT TCCCAGTCAC GACGTTGTAA AACGACGGCC
AGTGAAT

pGN59A FIG. 15.

GAGTGCACCA TATGCGGTGT GAAATACCGC ACAGATGCGT AAGGAGAAAA
TACCGCATCA GGCGAAATTG TAAACGTTAA TATTTTGTTA AAATTCGCGT
TAAATATTTG TTAAATCAGC TCATTTTTTA ACCAATAGGC CGAAATCGGC
AAAATCCCTT ATAAATCAAA AGAATAGACC GAGATAGGGT TGAGTGTTGT
TCCAGTTTGG AACAAGAGTC CACTATTAAA GAACGTGGAC TCCAACGTCA
AAGGGCGAAA AACCGTCTAT CAGGGCGATG GCCCACTACG TGAACCATCA
CCCAAATCAA GTTTTTTGCG GTCGAGGTGC CGTAAAGCTC TAAATCGGAA
CCCTAAAGGG AGCCCCCGAT TTAGAGCTTG ACGGGGAAAG CCGGCGAACG
TGGCGAGAAA GGAAGGGAAG AAAGCGAAAG GAGCGGGCGC TAGGGCGCTG
GCAAGTGTAG CGGTCACGCT GCGCGTAACC ACCACACCCG CCGCGCTTAA
TGCGCCGCTA CAGGGCGCGT CCATTCGCCA TTCAGGCTGC GCAACTGTTG
GGAAGGGCGA TCGGTGCGGG CCTCTTCGCT ATTACGCCAG CTGGCGAAAG
GGGGATGTGC TGCAAGGCGA TTAAGTTGGG TAACGCCAGG GTTTTCCCAG
TCACGACGTT GTAAAACGAC GGCCAGTGAA TTGTAATACG ACTCACTATA
GGGCGAATTC GAGCTCGGTA CCCGGGGATC CTCTAGAGAT CCCTCGACCT
CGAGATCCAT TGTGCTGGAA AGGATCTGGA TCCGGCTTAC TAAAAGCCAG
ATAACAGTAT GCGTATTTGC GCGCTGATTT TTGCGGTATA AGAATATATA
CTGATATGTA TACCCGAAGT ATGTCAAAAA GAGGTGTGCT ATGAAGCAGC
GTATTACAGT GACAGTTGAC AGCGACAGCT ATCAGTTGCT CAAGGCATAT
ATGATGTCAA TATCTCCGGT CTGGTAAGCA CAACCATGCA GAATGAAGCC
CGTCGTCTGC GTGCCGAACG CTGGAAAGCG GAAAATCAGG AAGGGATGGC
TGAGGTCGCC CGGTTTATTG AAATGAACGG CTCTTTTGCT GACGAGAACA
GGGACTGGTG AAATGCAGTT TAAGGTTTAC ACCTATAAAA GAGAGAGCCG
TTATCGTCTG TTTGTGGATG TACAGAGTGA TATTATTGAC ACGCCCGGGC
GACGGATGGT GATCCCCCTG GCCAGTGCAC GTCTCTTAAG CGATAAAGTC
TCCCGTGAAC TTTACCCGGT GGTGCATATC GGGGATGAAA GCTGGCGCAT
GATGACCACC GATATGGCCA GTGTGCCGGT CTCCGTTATC GGGGAAGAAG
TGGCTGATCT CAGCCACCGC GAAAATGACA TCAAAAACGC CATTAACCTG
ATGTTCTGGG GAATATAAAT GTCAGGCTCC CTTATACACA GCCTTTCCAG
CACAATGGAT CTCGAGGGAT CTTCCATACC TACCAGTTCT GCGCCTGCAG
GTCGCGGCCG CGACTCTCTA GAGTCGAAAG CTTCTCGCCC TATAGTGAGT
CGTATTACAG CTTGAGTATT CTATAGTGTC ACCTAAATAG CTTGGCGTAA
TCATGGTCAT AGCTGTTTCC TGTGTGAAAT TGTTATCCGC TCACAATTCC
ACACAACATA CGAGCCGGAA GCATAAAGTG TAAAGCCTGG GGTGCCTAAT
GAGTGAGCTA ACTCACATTA ATTGCGTTGC GCTCACTGCC CGCTTTCCAG
TCGGGAAACC TGTCGTGCCA GCTGCATTAA TGAATCGGCC AACGCGCGGG
GAGAGGCGGT TTGCGTATTG GGCGCTCTTC CGCTTCCTCG CTCACTGACT
CGCTGCGCTC GGTCGTTCGG CTGCGGCGAG CGGTATCAGC TCACTCAAAG
GCGGTAATAC GGTTATCCAC AGAATCAGGG GATAACGCAG GAAAGAACAT
GTGAGCAAAA GGCCAGCAAA AGGCCAGGAA CCGTAAAAAG GCCGCGTTGC
TGGCGTTTTT CGATAGGCTC CGCCCCCCTG ACGAGCATCA CAAAAATCGA
CGCTCAAGTC AGAGGTGGCG AAACCCGACA GGACTATAAA GATACCAGGC
GTTTCCCCCT GGAAGCTCCC TCGTGCGCTC TCCTGTTCCG ACCCTGCCGC
TTACCGGATA CCTGTCCGCC TTTCTCCCTT CGGGAAGCGT GGCGCTTTCT

FIG. 15 (CONTINUED)

CATAGCTCAC GCTGTAGGTA TCTCAGTTCG GTGTAGGTCG TTCGCTCCAA
GCTGGGCTGT GTGCACGAAC CCCCCGTTCA GCCCGACCGC TGCGCCTTAT
CCGGTAACTA TCGTCTTGAG TCCAACCCGG TAAGACACGA CTTATCGCCA
CTGGCAGCAG CCACTGGTAA CAGGATTAGC AGAGCGAGGT ATGTAGGCGG
TGCTACAGAG TTCTTGAAGT GGTGGCCTAA CTACGGCTAC ACTAGAAGGA
CAGTATTTGG TATCTGCGCT CTGCTGAAGC CAGTTACCTT CGGAAAAAGA
GTTGGTAGCT CTTGATCCGG CAAACAAACC ACCGCTGGTA GCGGTGGTTT
TTTTGTTTGC AAGCAGCAGA TTACGCGCAG AAAAAAAGGA TCTCAAGAAG
ATCCTTTGAT CTTTTCTACG GGGTCTGACG CTCAGTGGAA CGAAAACTCA
CGTTAAGGGA TTTTGGTCAT GAGATTATCA AAAAGGATCT TCACCTAGAT
CCTTTTAAAT TAAAAATGAA GTTTTAAATC AATCTAAAGT ATATATGAGT
AAACTTGGTC TGACAGTTAC CAATGCTTAA TCAGTGAGGC ACCTATCTCA
GCGATCTGTC TATTTCGTTC ATCCATAGTT GCCTGACTCC CCGTCGTGTA
GATAACTACG ATACGGGAGG GCTTACCATC TGGCCCCAGT GCTGCAATGA
TACCGCGAGA CCCACGCTCA CCGGCTCCAG ATTTATCAGC AATAAACCAG
CCAGCCGGAA GGGCCGAGCG CAGAAGTGGT CCTGCAACTT TATCCGCCTC
CATCCAGTCT ATTAATTGTT GCCGGGAAGC TAGAGTAAGT AGTTCGCCAG
TTAATAGTTT GCGCAACGTT GTTGGCATTG CTACAGGCAT CGTGGTGTCA
CGCTCGTCGT TTGGTATGGC TTCATTCAGC TCCGGTTCCC AACGATCAAG
GCGAGTTACA TGATCCCCCA TGTTGTGCAA AAAAGCGGTT AGCTCCTTCG
GTCCTCCGAT CGTTGTCAGA AGTAAGTTGG CCGCAGTGTT ATCACTCATG
GTTATGGCAG CACTGCATAA TTCTCTTACT GTCATGCCAT CCGTAAGATG
CTTTTCTGTG ACTGGTGAGT ACTCAACCAA GTCATTCTGA GAATACCGCG
CCCGGCGACC GAGTTGCTCT TGCCCGGCGT CAATACGGGA TAATAGTGTA
TGACATAGCA GAACTTTAAA AGTGCTCATC ATTGGAAAAC GTTCTTCGGG
GCGAAAACTC TCAAGGATCT TACCGCTGTT GAGATCCAGT TCGATGTAAC
CCACTCGTGC ACCCAACTGA TCTTCAGCAT CTTTTACTTT CACCAGCGTT
TCTGGGTGAG CAAAAACAGG AAGGCAAAAT GCCGCAAAAA AGGGAATAAG
GGCGACACGG AAATGTTGAA TACTCATACT CTTCCTTTTT CAATATTATT
GAAGCATTTA TCAGGGTTAT TGTCTCATGA GCGGATACAT ATTTGAATGT
ATTTAGAAAA ATAAACAAAT AGGGGTTCCG CGCACATTTC CCCGAAAAGT
GCCACCTGAC GTCTAAGAAA CCATTATTAT CATGACATTA ACCTATAAAA
ATAGGCGTAT CACGAGGCCC TTTCGTCTCG CGCGTTTCGG TGATGACGGT
GAAAACCTCT GACACATGCA GCTCCCGGAG ACGGTCACAG CTTGTCTGTA
AGCGGATGCC GGGAGCAGAC AAGCCCGTCA GGGCGCGTCA GCGGGTGTTG
GCGGGTGTCG GGGCTGGCTT AACTATGCGG CATCAGAGCA GATTGTACTG
A

FIG. 16.

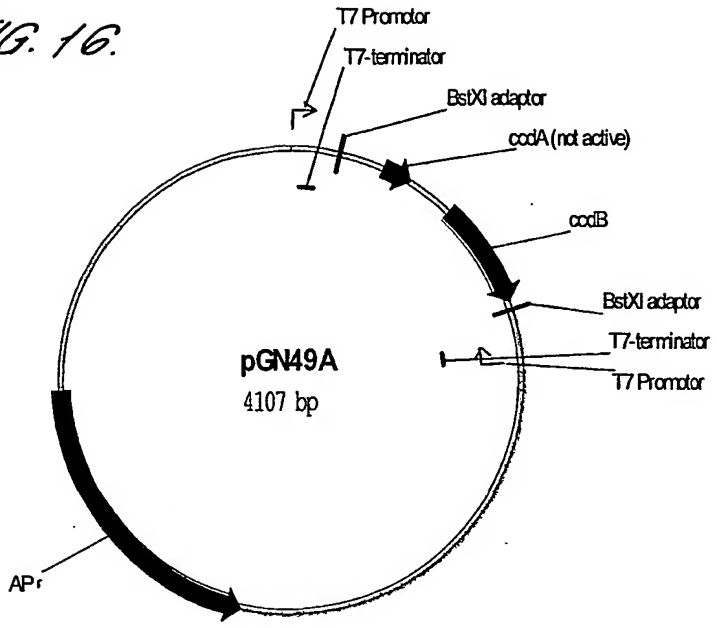
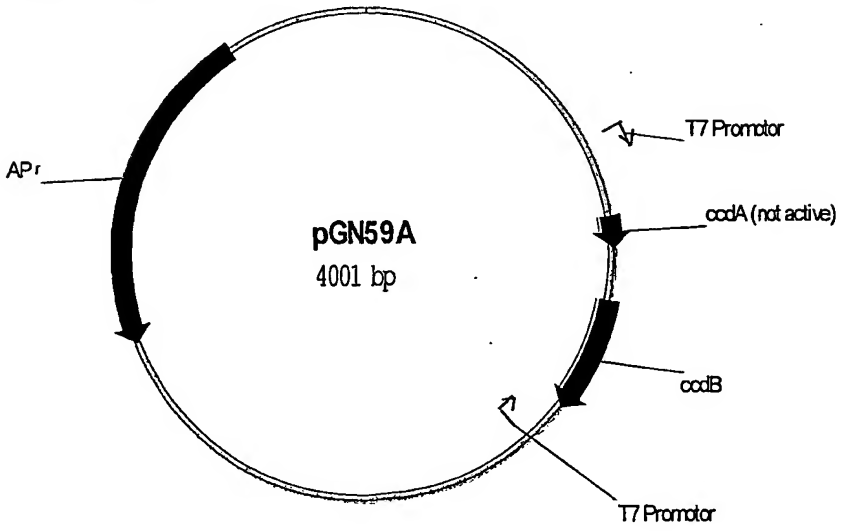

T7 Promotor
T7-terminator
BstXI adaptor
ccdA (not active)
ccdB
BstXI adaptor
T7-terminator
T7 Promotor
pGN49A
4107 bp
APr

FIG. 17.


APr
T7 Promotor
ccdA (not active)
pGN59A
4001 bp
ccdB
T7 Promotor

SEQUENCE LISTING

<110> DEVGEN NV

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<130> SCB/55178/001

<140>
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<160> 21

<170> PatentIn Ver. 2.0

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<213> Artificial Sequence

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<223> Description of Artificial Sequence: Fragment of
pGN1 containing opposable T7 promoters

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<210> 2
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<223> Description of Artificial Sequence: DNA sequence
containing a T7 terminator

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<223> Description of Artificial Sequence:
Oligonucleotide oGN27

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<223> Description of Artificial Sequence:

Oligonucleotide oGN28

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<223> Description of Artificial Sequence:
Oligonucleotide oGN29

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<223> Description of Artificial Sequence:
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<223> Description of Artificial Sequence: Fragment of
plasmid pGN9 containing opposable T7 promoters and
T7 transcription terminators

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<223> Description of Artificial Sequence: Plasmid pGN29

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<223> Description of Artificial Sequence: Plasmid pGN39

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<210> 11
<211> 3715
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Plasmid
TopoRNAi

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<210> 12
<211> 4107
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Plasmid pGN49A

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<210> 13
<211> 4001
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Plasmid pGN59A

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<210> 14
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
Oligonucleotide oGN103

<400> 14
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<210> 15
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
Olgigonucleotide oGN104

<400> 15
taccaaggct agcatgggcc tgcctgaagg ctgc 34

<210> 16
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
Oligonucleotide oGN126

<400> 16
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<210> 17

<211> 46
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
Oligonucleotide oGN127

<400> 17
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<210> 18
<211> 51
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
Oligonucleotide oGN128

<400> 18
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<210> 19
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
Oligonucleotide oGN129

<400> 19
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<210> 20
<211> 375
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR fragment
generated by primers oGN103 and 0GN104 on pCDM8

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ccctggactg ttgaacgagg tcggcgtaga cggtctgacg acacgcaaac tggcggaacg 180
gttgggggtg cagcagccgg cgctttactg gcacttcagg aacaagcggg cgctgctcga 240
cgcactggcc gaagccatgc tggcggagaa tcatacgctt cggtgccgag agccgacgac 300
gactggcgct catttctgat cgggaatccc gcagcttcag gcaggcccat gctagccttg 360
gtaccagcac aatgg 375

<210> 21
<211> 670
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR fragment

<400> 21
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gcggtataag aatatatact gatatgtata cccgaagtat gtcaaaaaga ggtgtgctat 120
gaagcagcgt attacagtga cagttgacag cgacagctat cagttgctca aggcatatat 180
gatgtcaata tctccggtct ggtaagcaca accatgcaga atgaagcccg tcgtctgcgt 240
gccgaacgct ggaaagcgga aaatcaggaa gggatggctg aggtcgcccg gtttattgaa 300
atgaacggct cttttgctga cgagaacagg gactggtgaa atgcagttta aggtttacac 360
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gcccgggcga cggatggtga tccccctggc cagtgcacgt ctcttaagcg ataaagtctc 480
ccgtgaactt tacccggtgg tgcatatcgg ggatgaaagc tggcgcatga tgaccaccga 540
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aaatgacatc aaaaacgcca ttaacctgat gttctgggga atataaatgt caggctccct 660
tatacacagc 670

INTERNATIONAL SEARCH REPORT

tional Application No
PCT/IB 01/01068

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/10 C12N15/63 C12N15/70 C12N1/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FR 2 782 325 A (PROTEUS) 18 February 2000 (2000-02-18) page 7, line 20 -page 8, line 8 page 11, line 11 - line 36 page 23, line 31 -page 24, line 9 ---	1-24,26, 27
A	WO 00 01846 A (DEVGEN N.V.) 13 January 2000 (2000-01-13) cited in the application page 8, line 9 -page 10, line 22 page 15, line 9 - line 33 page 21, line 21 -page 22, line 29 --- -/--	1-28

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 September 2001	27/09/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Montero Lopez, B

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INTERNATIONAL SEARCH REPORT

	national Application No PCT/IB 01/01068

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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